DOCTORAL THESIS

An investigation into the distribution of obligate groundwater animals (stygobites) in England and Wales

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An investigation into the distribution of obligate groundwater animals (stygobites) in England and Wales

N. kochianus captured from the Chalk in England, UK.

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II Abstract

Aquifers and their biota (stygobites) are some of the least well known ecosystems. Previous research indicates geology is an important control of stygobites and the primary thesis aim was therefore to examine how geology shapes the distributions of stygobites across different spatial scales, considering high lithological detail. The food web functionality of groundwater ecosystems is also rarely explored and the secondary thesis aim was therefore to examine the trophic effects of stygobites in experimental groundwater microcosms. The new habitat typology shows that 11 higher resolution geo-habitats are characterised by significantly different hydrochemistry and a heterogeneous distribution of high-quality habitat patches. Furthermore, the habitat scoring system developed based on the variability of influential abiotic parameters shows that overall geo-habitat quality varies considerably, with karstic geo-habitats (e.g. Chalk) having a higher quality than most porous and fractured geo-habitats. Major parts of England and Wales are covered by poor quality habitats, probably limiting dispersal. Testing the new typology on species distributions shows that biodiverse and / or abundant communities occur in all geo-habitats. Karstic aquifers generally form the best habitats with the highest stygobite species diversity and frequency of occurrence. However, some fractured aquifers (e.g. Igneous Rock) are also significant habitats for stygobites, while more geological detail needs to be considered to explain stygobite communities in other fractured aquifers (e.g. Mudstones & Siltstones). However, many species are not distributed throughout entire connected aquifers, and in individual sites copepod biomass is not explained by geology, showing that other environmental controls (glacial history, site characteristics) may supersede geological controls. The grazing experiments show

that stygobites significantly increase protozoan abundance and morphotype diversity, and alter microbial community structure, indicating they may modulate the ecosystem services provided by these groups. Overall, this thesis enhances our knowledge of the factors governing the distribution and functionality of groundwater ecosystems.

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IV Acknowledgments

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Chapter 1 – Introduction

1.1 Summary of thesis aims:

The main aim of this thesis was to improve our understanding of the distribution of groundwater ecosystems in England and Wales, with a particular focus on how this is shaped by geological controls.

The first chapter provides a general introduction to groundwater, its ecosystems and the importance of geology in controlling groundwater communities (sections 1.2 – 1.4). It then identifies important knowledge gaps pertaining to groundwater ecosystems (sections 1.5 – 1.9) and provides context for the work undertaken in each of the following chapters in the thesis.

The aim of the second chapter was to develop a new geological habitat (geo-habitat) typology for England and Wales, and to determine the differences in abiotic characteristics between the geo-habitats. Furthermore, a system of quality scores was developed to characterise the potential suitability of these habitats to groundwater ecosystems. Overall, it was hypothesised that karstic rocks would provide more suitable geo-habitats than porous and fractured rocks.

The main aim of chapter three was to undertake field sampling and combine the results with previous data to assess the groundwater ecology of four geologies, which had either not been sampled previously (Devonian Old Red Sandstone, Lower Greensand) or had considerable gaps in sampling coverage (Jurassic Limestone, Magnesian Limestone). An additional aim was estimate faunal biomass in the four geologies, and to assess whether geology or other environmental factors control copepod biomass and

abundance in groundwater. Amphipod stygobites were too rare to enable an analysis of this. It was hypothesised that the limestones would harbour higher stygobite (and copepod) diversity, abundance and biomass than the sandstones. However, it was also expected that determinants other than geology (e.g. glacial history, dispersal abilities of different species) would influence stygobite distributions and copepod response variables.

Using the geo-habitat typology developed in chapter two as a framework, the new stygobite data collected for chapter three, and records from other studies, the aims of chapter four were to evaluate whether habitats differ in their ecological communities, and to determine whether the distributions of different stygobite species are controlled by geology. It was hypothesised that karstic geo-habitats would harbour higher species diversity and abundance than fractured geo-habitats. Furthermore, it was expected that while geology controls species distributions to some extent, further species-specific determinants (e.g. species' dispersal ability) would often be more important.

The aim of chapter five was to investigate the trophic effects of several stygobite species on bacteria and protozoa in two complementary laboratory experiments. It was hypothesised that stygobites would affect both protozoan and microbial diversity and abundance, and that these grazing effects would vary with grazer species, grazer density and the duration of experiments.

Finally, chapter six summarises the most important results in a synthesis, which identifies the overarching themes from the four chapters, highlights the most significant findings and presents remaining knowledge gaps that should be addressed in future research.

1.2 Groundwater Characteristics:

'Water is the driver of nature – Leonardo da Vinci'

Although water covers 71% of the earth's surface, 96.5% of this occurs as saltwater in seas and oceans. Freshwater, which is an essential resource for most organisms, makes up only 2.5% of the earth's total water volume, of which 68.7 % is immobilised in ice caps and glaciers (Gleick, 1993). Of the liquid freshwater only 0.3% is held in surface water bodies and the atmosphere, while the remainder circulates through soils and rock formations as groundwater (UNEP Report, 1995).

Thus groundwater is of great importance to both the earth's ecosystems and to humans. Numerous hydrogeological definitions of the term 'groundwater' exist. For example, Freeze & Cherry (1979) defined groundwater as: *'Water beneath the water table in soils and geological formations that are fully saturated'*. However, as water moves through aquifers it is modified by abiotic and biotic processes, resulting in a range of different habitats in time and space. These changes are likely to have consequences for groundwater-dependent ecosystems as well as the human consumption of groundwater, and so a more inclusive definition acknowledging the ecosystem component may be: *'Groundwater is water that has been present in pores and cracks of the saturated zone of soil or rock for sufficient time to undergo physical and chemical changes resulting from interactions with the aquifer environment'* (Tomlinson & Boulton, 2008).

A fundamental determinant of groundwater and its associated ecosystems is the connectivity to surface ecosystems. Exchanges of water, materials and organisms between aquifers and surface ecosystems take place at the interfaces between these

systems along dynamic gradients (Tomlinson & Boulton, 2008) (Fig. 1.1). The infiltrating surface water replenishes nutrients, organic matter and oxygen, but may also bring in agricultural and industrial contaminants, such as nitrate. Nutrient and oxygen replenishment is dependent on a multitude of abiotic factors, including temperature, precipitation, humidity and human influences such as the alteration of hydraulic gradients in the subsurface (Ward & Robinson, 1990). Groundwater communities vary with distance from the nearest surface water body; obligate subterranean animals (stygobites) dominate communities in soil-recharged groundwater ecosystems but they may be less dominant where surface water bodies allow the influx of surface species (Fig. 1.1) (Schmidt & Hahn, 2012). Groundwater moves horizontally at varying velocities from several millimetres per year to several kilometres per day in karst aquifers (Ford & Williams, 1989; Maurice et al., 2006) towards an outlet from the aquifer, such as another surface water body (Fig. 1.1). Underground the hydrochemical composition of water is modified by geochemical reactions with bedrock and the activity of microbes and / or invertebrates, and the extent of modification depends on the water's flow velocity.

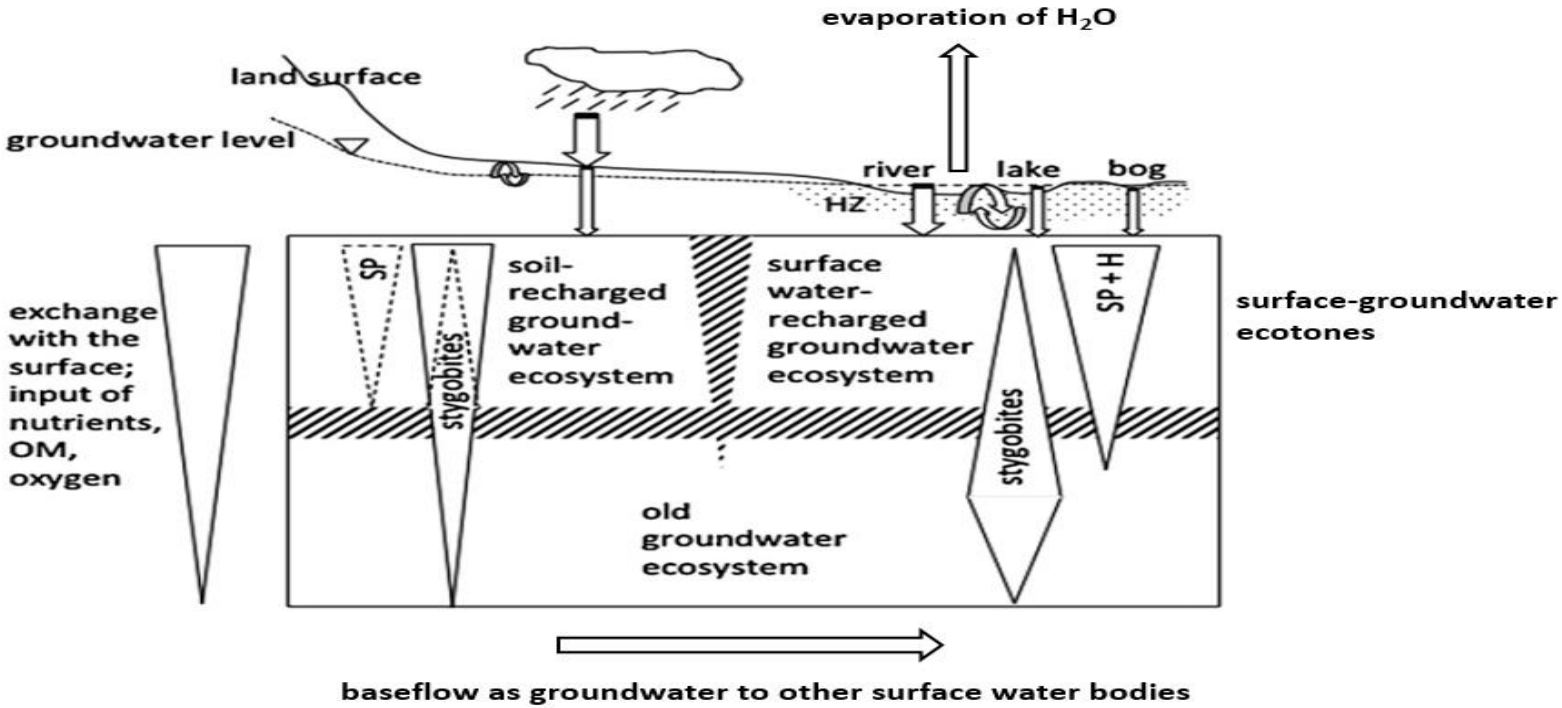


Fig. 1.1: Diagram illustrating the hydrological cycle and the hydrological interactions between surface (soil and water) and groundwater ecosystems along ecotonal boundaries. The effects of hydrological interactions on nutrients, organic matter (OM), oxygen and communities are indicated by triangles and diamonds (lowest values at the tips). Nutrients and oxygen levels decrease with depth in both scenarios. In soil-recharged groundwater ecosystems stygobites (obligate groundwater animals) tend to dominate at all depths, while groundwater ecosystems under strong influence from surface water bodies are dominated by SP (stygophiles) and H (hyporheic taxa) near the ecotone with stygobites becoming dominant with depth. Groundwater continues horizontally as baseflow towards aquifer outlets, being continuously modified by geochemical and microbial processes. (adapted from Schmidt & Hahn, 2012)

With rare exceptions such as cave entrances or open boreholes, most groundwater habitats are completely dark (Tomlinson & Boulton, 2008). There are no photosynthesizing primary producers and groundwater ecosystems are entirely dependent on allochthonous energy sources, such as particulate organic carbon (POC) or dissolved organic carbon (DOC). This leads to very truncated food webs that are dominated by detritivores (Gibert & Deharveng, 2002). Groundwater food webs are composed of few low-abundance trophic levels. Microbes form the basal trophic level and assimilate infiltrating carbon and nutrients. They are fed upon by a diverse array of protozoans and invertebrates, which are usually the top consumers in the subsurface, although fish may be present in cave systems. Energy input into groundwaters is low and resource scarcity is one of the main controls limiting population abundances of groundwater fauna (Datry et al., 2005). As a result, groundwater fauna has evolved lower metabolic rates, longer life cycles and lower fecundity than related surface species (Gibert et al., 1994; Dole-Olivier et al., 2000) and thus may be particularly vulnerable to anthropogenic disturbances, such as contaminant pollution or changing temperatures (Danielopol et al., 2003; Robertson et al., 2009). Due to similar environmental pressures in all groundwater habitats, resident species display high morphological convergence (Cooper et al., 2007; Finston et al., 2007; Humphreys, 2008) and molecular analyses are often needed to identify separate lineages (Lefébure et al., 2007).

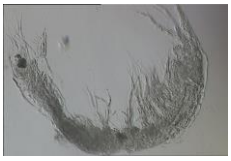
Groundwater habitats tend to be spatially discrete and patchy (Dole-Olivier et al., 2009a; Larned, 2012). Geological formations exhibit extreme heterogeneity, resulting in spatial variability of many habitat characteristics such as hydrochemistry. For example, some rocks may be characterised by unconsolidated material and high permeability in one location, while being cemented and having low permeability in a nearby area (Allen

et al., 1997). Permeability and hydrological continuity are very important factors for groundwater ecosystems because they determine the distribution and dispersal capacity of groundwater fauna (Hahn, 2006; Stein et al., 2012; Maurice et al., 2015). Groundwater habitat heterogeneity is the main reason for the occurrence of high rates of speciation and short-range endemism in many regions, such as in isolated cave systems of Slovenia and North America (Culver & Sket, 2000). Lithological features, such as voids in karstic systems that allow the rapid infiltration of contaminants (Cook et al., 2012), are also the reason why groundwater fauna are thought to be particularly vulnerable to habitat loss and extinction events (Strayer, 1994; Proudlove et al., 2003). However, despite the local endemism and the proposed vulnerability of groundwater fauna, some faunal lineages have persisted for over 19 million years and have relatively broad geographical distributions (McInerney et al., 2014).

The term 'groundwater animal' describes any organism that is found in groundwater. However, such animals differ in their degree of dependency on groundwater. Three different groups have been defined by Gibert et al. (1994): Stygoxenes occur accidentally in groundwater, but may survive there temporarily. Stygophiles occur in groundwater on a temporary basis, generally invading when environmental conditions are suitable. Obligate groundwater inhabitants (stygobites) complete their entire life cycle in groundwater. Globally, stygobites belong largely to the phylum Crustacea and have been estimated at 7700 species (Deharveng et al., 2009). Cryptic speciation is very prevalent in the groundwater environment, resulting in morphologically similar but genetically very distinct species and thus increasing use of molecular analyses will undoubtedly result in the discovery of more species (Lefebure et al., 2007; Niemiller et al., 2013; McInerney et al., 2014).

In Britain there are only eight recorded stygobite species, all of which belong to the subphylum Crustacea and mostly to the order Amphipoda (see Table 1.1 for a list). However, many more are found in the biodiversity hotspots of the Dinaric karst in eastern Europe (e.g. Culver & Sket, 2000), central Europe (Gibert et al., 2009) and some areas of the USA (Christman et al., 2005) and Australia (78 recorded species, Eberhard et al., 2005). In the UK and other northern European countries, repeated glacial episodes have probably led to widespread extinctions, resulting in the low observed species diversity (e.g. Proudlove et al., 2003; Castellarini et al., 2007; Robertson et al., 2009; Dole-Olivier et al., 2009; Galassi et al., 2009; McInerney et al., 2014). Only few stygobites have been recorded in previously glaciated areas and are likely from species (*A. stammeri*) that have survived in deep subsurface refugia where conditions remained relatively stable (Proudlove et al., 2003). The low stygobite diversity may make groundwater ecosystems in northern Europe more vulnerable to natural disturbances or human-induced pollution because ecosystems with few components have less redundancy than more complex ones, and are thus more likely to collapse with species loss (Lawton, 1994; Naeem & Li, 1997; Reich et al., 2012; Micheli et al., 2014).

Table 1.1: Species list of obligate groundwater animals (stygobites) in England and Wales.

Stygobite Species (described by)	Order	Photo
Crangonyx subterraneus (Bate, 1859)	Amphipoda	 1
Microniphargus leruthi (Schellenberg, 1934)	Amphipoda	 2
Niphargus aquilex (Schiodte, 1855)	Amphipoda	 3
Niphargus fontanus (Bate, 1859)	Amphipoda	 4
Niphargus glenniei (Spooner, 1952)	Amphipoda	 5
Niphargus kochianus kochianus (Schellenberg, 1932)	Amphipoda	 6
Proasellus cavaticus (Leydig, 1871 sensu Henry, 1970)	Isopoda	 7
Anthrobathynella stammeri (Jakobi, 1954)	Bathynellacea	 8

1 Jessica Durkota, 2 hcrs.freshwaterlife.org, 3, 5 Chris Proctor, 4 Phil Chapman, 6 Koorosh McKormack, 7 Damiano Weitowitz, 8 Johns & Dunscombe (2011)

1.3 Conservation status of groundwater ecosystems:

Stygobites make a unique contribution to biodiversity and are parts of ecosystems that contribute to groundwater purification, the attenuation of pollutants and the long-term storage of drinking water (Tomlinson & Boulton, 2010; Griebler & Avramov, 2015). Furthermore, groundwater organisms, crustaceans and microbes, may be used as indicators for the chemical and ecological status of groundwater (Malard et al., 1996; Steube et al., 2009; Stein et al., 2010; Korbel & Hose, 2011). Despite this, ecological perspectives are rarely incorporated into groundwater management policy (Danielopol et al., 2004). The groundwater policies that have been formulated in the Water Framework Directive (WFD, Council of the European Communities, 2000) and the EU Groundwater Directive (GWD, Council of the European Communities, 2006) largely deal with the abiotic status of groundwater, but have called for further research on groundwater ecosystems. Currently only the stygobite species *N. glenniei*, endemic to south-west England, is officially protected as part of the UK Biodiversity Action Plan (BAP, 2007), mainly due to its small geographic distribution range (Knight, 2009). However, there are other species that may also require protection. For example, *A. stammeri* is a rare stygobite that is only known from a few sites in England. Furthermore, *N. kochianus* is largely restricted to a single aquifer, the Chalk (Maurice et al., 2015), and may be at potential risk by habitat degradation. Globally, biodiversity is lost at alarming rates due to a multitude of interacting factors, ranging from natural processes to anthropogenic effects (Dudgeon et al., 2006). In order to identify species diversity hotspots and to understand the factors governing this diversity it is imperative to understand spatial variation in diversity and abundance. Overall, further research into the functioning of groundwater ecosystems is clearly needed to inform the legislative bodies of the government.

1.4 Geology – A control across multiple scales

Groundwater communities are controlled by a multitude of interacting factors, operating across a range of temporal and spatial scales (Gibert et al., 1994; Hahn, 2009; Stoch & Galassi, 2010; Maurice & Bloomfield, 2012) (Fig. 1.2). Large-scale controls (100s – 1000s km) shaping groundwater communities operate in deep geological time, including past glaciations (Kristjánsson & Svavarsson, 2007; Robertson et al., 2009), geological history (Notenboom, 1991), plate tectonics (Schminke, 1974) and marine transgressions (Illies, 1967; Jaume & Humphreys, 2001) (Fig. 1.2). At the mid-scale (10s km) groundwater assemblages are often shaped by the type of geology, such as compact, porous and fractured rocks (Hahn & Fuchs, 2009; Johns et al., 2015) (Fig. 1.2), where the highest diversity and abundances are found in karstic and porous rocks (Dole-Olivier et al., 2009a; Hahn & Fuchs, 2009). At the local-scale (< 1 km) water chemistry, which is mostly dependent on surface connectivity, is thought to influence species distributions (Dumas et al., 2001; Datry et al., 2005; Hahn, 2006) (Fig. 1.2). DO and DOC concentrations were positively associated with stygobite diversity and abundance in various studies (Malard et al., 2003; Datry et al., 2005; Hahn, 2006; Dole-Olivier et al., 2009a; Griebler et al., 2010; Stein et al., 2010).

Geology, a mid-scale control, directly determines the physical habitat available to invertebrates. Karstic rocks (e.g. limestones) with large voids or caves and unconsolidated rocks (e.g. gravel) with large pore spaces offer more habitat space to resident communities. Larger species may be excluded from habitats that lack appropriately sized voids or pore spaces to move through. Past studies have found that rocks with larger void spaces support more diverse and abundant communities (Hahn & Fuchs, 2009; Johns et

al., 2015; Maurice et al., 2015). The faunal community complexity within aquifers will also depend on the connectivity of physical habitat features, such as fractures, voids and caves.

Lithology also determines the permeability or transmissivity and thus hydrochemical conditions in the rock. Rocks with large voids or pore spaces are more permeable and have increased input of nutrients and oxygen from the surface (Hahn, 2006). Additionally, water reacts with rock, continuously dissolving ions into surrounding water. For example, dissolution of calcium carbonate leads to very high levels of dissolved calcium in groundwater of karstic rocks (Price, 1985). Although comparatively few studies have investigated the chemical requirements of stygobites, most have found that spatial chemical variation accounts for at least part of the observed species distributions (e.g. Hahn, 2006; Dole-Olivier et al., 2009a). The local-scale variation of water chemistry is therefore also determined by geology, and nested within the mid-scale control (Fig. 1.2, B).

Some of the hydrochemical drivers of stygobites (e.g. dissolved oxygen and calcium) affect stygobites directly, whereas others (e.g. dissolved organic carbon and nitrate) exert their influence via indirect bottom-up cascades through the groundwater food chain. For example, stygobites, like other crustaceans (e.g. Lasker, 1966), need free oxygen to obtain energy from oxidising organic compounds. Although stygobites are known to have lower metabolic rates than surface species (Wilhelm et al., 2006) and have been recorded in suboxic habitat patches (Malard & Hervant, 1999), several studies have highlighted that they are partly controlled by oxygen gradients (e.g. Malard et al., 2003; Datry et al., 2005). In contrast, nitrate, an indirect bottom-up fuel for stygobites, is only

likely to have a significant effect in oxygen-depleted aquifers (Rivett et al., 2008). In aerobic conditions, groundwater bacteria preferentially use oxygen as an electron acceptor for oxidation processes, which is replaced by nitrate once the oxygen is consumed. While this redox sequence is observed in most aquifers (e.g. Edmunds et al., 1982; Christensen et al., 2000), multiple redox reactions may occur simultaneously in aquifers (McGuire et al., 2002). Furthermore, interpreting the interactions between hydrochemical predictors and faunal assemblages is very complex. While increasing nutrient concentrations, either carbon or nitrate, below critical thresholds are likely to increase faunal abundance and / or diversity (e.g. Datry et al., 2005), excessive nutrient pollution is likely to trigger stygobite disappearance (Hahn, 2006; Galassi et al., 2009a; Schmidt & Hahn, 2012).

Furthermore, the hydrological connectivity of different geologies influences the dispersal capacity of groundwater fauna. Groundwater habitats tend to be very fragmented and limit the dispersal of subterranean animals, which is likely to be the reason for the low post-glacial recolonisation of stygobites in northern Europe (Proudlove et al., 2003; Lefebure et al., 2007; Robertson et al., 2009). The heterogeneity of rocks therefore reinforces glacial distribution controls that operate on the macro-scale (Fig. 1.2, A). Low-permeability rocks may also limit species mobility elsewhere, such as mudstones in south-west England which may constrain the distribution of *N. glenniei* (Johns et al., 2015).

The importance of geology as a control influencing groundwater ecosystems at all spatial scales (Fig. 1.2) is a recurrent theme in this introduction and in the following data chapters.

1.5 Current habitat typologies

To explain the distribution of communities at the mid- and large-scale (e.g. at a national level) a habitat typology is needed, to allow a comparison of communities between proposed habitat types. Generally, habitat classifications are an important component of planning sampling efforts, designing monitoring programmes and prioritising sites for conservation action (Maddock, 1999; Eroes, 2007; Larned, 2012), and often form the basis of research hypotheses and sampling surveys (Hahn, 2009). Developing an integrative typology, based on relevant criteria for groundwater fauna, is a necessary prerequisite for understanding what drives currently known species distributions and in predicting faunal assemblages in previously unsampled areas. As is done in surface waters, studies can then follow to evaluate whether the observed communities are in line with the predictions. Such directed surveying work is essential in supporting conservation policy and may be an important step towards including groundwater ecosystem assessments in future iterations of the EU Groundwater Directive.

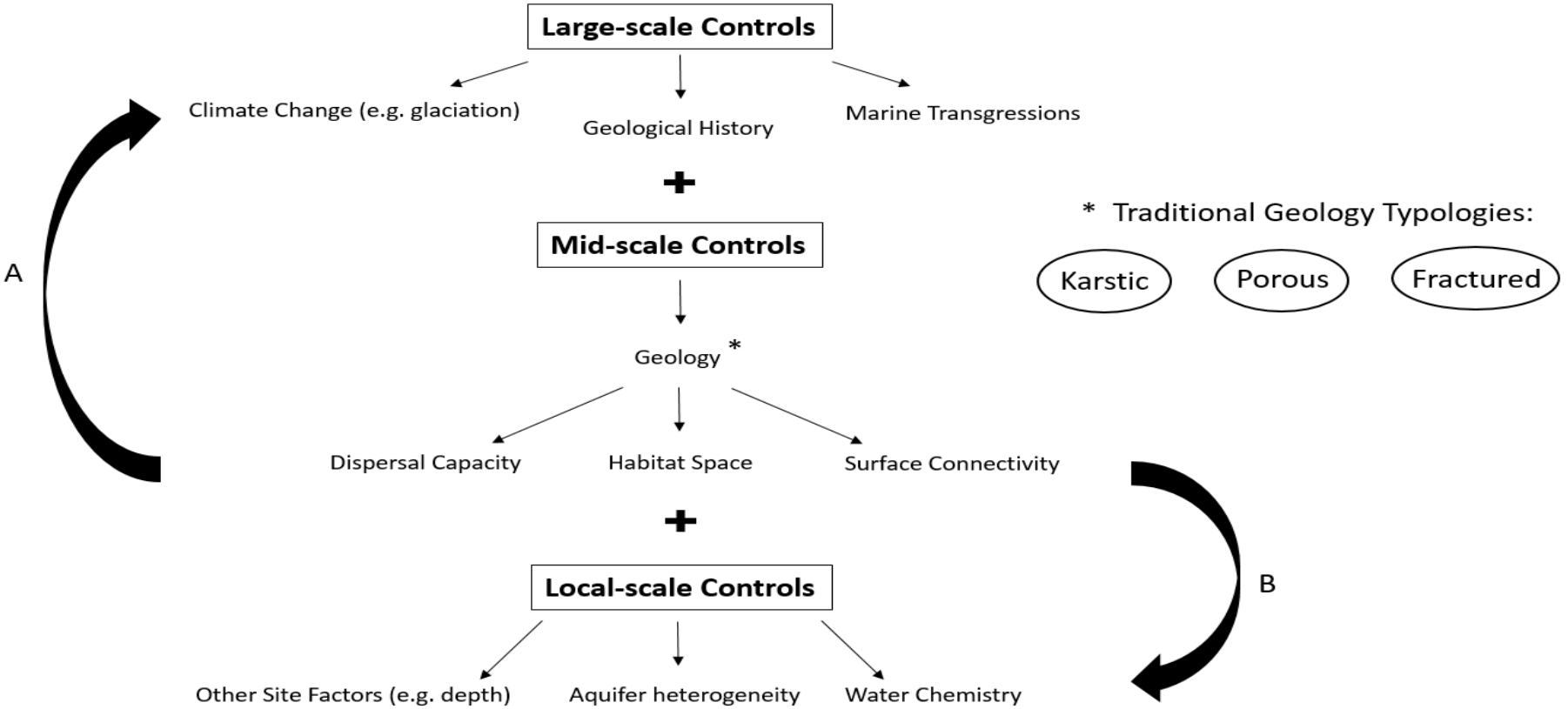


Fig. 1.2: Diagram of some of the most important environmental factors controlling the observed stygobite distributions, with particular emphasis on the role of geology. Shown are large-scale, mid-scale and local-scale controls. Geology determines three key features of groundwater habitats, including habitat space, surface connectivity and faunal dispersal capacity. These effects feed back into large-scale controls such as glacial controls (A) and local-scale controls such as water chemistry (B). * In the traditional assessment of geological controls rocks have been grouped into karstic, porous and fractured rocks. (adapted from Maurice & Bloomfield, 2012)

Most habitat classifications are hierarchical, in which numerous small units are nested within fewer more inclusive ones (Frissell et al., 1986; Larned, 2012). Broad categories provide information on large-scale distribution trends, while high resolution categories provide explanatory power at fine spatial scales. Frissell et al., (1986) developed a candidate classification grouping surface water ecosystems at various levels, ranging from the broad system to the microhabitat level. For groundwater ecosystems similar hierarchical frameworks have been suggested, although most have not been tested with ecological data (Larned, 2012). However, species distributions are not only influenced across different spatial scales, but also by environmental variability within a given scale (e.g. geology, mid-scale control, Fig. 1.2).

Existing groundwater habitat classifications are relatively broad, distinguishing between broad aquifer types such as karstic, fractured and porous (Dole-Olivier et al., 2009a; Galassi et al., 2009a; Hahn & Fuchs, 2009). Most studies using this approach found significant differences in groundwater communities, indicating that a lithology-based typology is a good starting point. However, using a broad resolution increases within-habitat variability and potentially decreases the explanatory power of a framework. There are a vast number of different geological units (Allen et al., 1997; Jones et al., 2000), so grouping into more inclusive categories is necessary to test hypotheses of species distributions.

Generally, aquifers are highly complex and provide heterogeneous habitats to groundwater fauna (Allen et al., 1997). These heterogeneities occur at different spatial scales, ranging from large-scale lithological variation across 1000's of metres to small-scale variation in interstitial pore spaces and fractures across 10^{-2} metres (Larned, 2012).

This variability is highly relevant for groundwater life, because it determines the available space, permeability, oxygen and nutrient distributions and dispersal pathways (Murphy et al., 1997; Goldscheider et al., 2006). Modelling of groundwater flow has shown that specific fractures or voids represent preferential flow paths, accounting for the majority of solute transport and dispersal (Shoemaker et al., 2008) and are likely to harbour the most diverse ecosystems. Although grouping of fractured geologies may be legitimate on their shared morphological features (i.e. fractures), the size and density of these may vary considerably. For example, the Aylesbeare mudstone in south-west England has few fractures with severely reduced water flow (Jones et al., 2000), while Devonian Old Red Sandstone is more densely fractured with higher permeability (Jones et al., 2000). In a broad typology these would be grouped together, omitting differences that are likely to be relevant for fauna.

More geological detail was incorporated in a distribution study investigating the complex geological setting of south-west England (Johns et al., 2015). Lithological and hydrological information was used to categorise rock types into 5 different hydro-units, including granular, igneous / metamorphic, mudstone / siltstone, sandstone and carbonate habitats. The typology predicted the presence of stygobites in some habitats, with stygobites being significantly less likely to be recorded in mudstones / siltstones and sandstones than in carbonate, granular or igneous / metamorphic habitats. Johns et al. (2015) thereby demonstrated that higher resolution typologies may explain faunal distributions more appropriately at regional scales.

Recently a European groundwater study established 13 habitats based on void size, flow type and permeability (Cornu et al., 2013), incorporating quantitative hydrogeological data to distinguish the habitats. In this study, Cornu et al. (2013) demonstrate that habitat and species diversity decrease with increasing latitude, providing evidence for a footprint of past glaciations on species distributions. The map proposed by Cornu et al. (2013) is a significant advance to a more integrative classification of groundwater habitats called for by researchers (e.g. Larned, 2012). However, at the country scale more geological detail may be required, because, as indicated by the authors, small species-rich areas may have escaped the relatively low spatial resolution. For example, igneous and metamorphic rocks in south-west England were classified as non-aquiferous rock with low suitability for groundwater fauna (Cornu et al., 2013), although they provide significant habitats for stygobites (e.g. the endemic species *N. glenniei*; Johns et al., 2015; Proudlove et al., 2003; Robertson et al., 2009). Finally, Cornu et al. (2013) and other studies have also called for the incorporation of hydrochemistry in habitat classifications, as this is thought to be a main determinant of groundwater communities (Dole-Olivier et al., 2009a; Larned, 2012; Cornu et al., 2013).

Overall, developing a typology with more geological detail and the support of quantitative hydrogeological and hydrochemical data provides the highest explanatory power for faunal distributions. Such a candidate classification would also feed into explaining distributions at multiple scales, ranging from the small-scale to the large-scale level (Fig. 1.2). In chapter two a national-scale habitat typology for England and Wales is developed for the first time, providing a framework for the species distribution assessment in chapter four and possibly future groundwater ecosystem investigations.

Additionally, a habitat quality scoring system is proposed, which could be applied and / or modified in forthcoming studies.

1.6 A global sampling bias

In addition to an appropriate habitat typology, thorough sampling in each of the habitats is needed to undertake ecosystem assessments. Good sampling coverage is particularly important in groundwater habitats, which display extreme heterogeneity at all spatial scales and consequently have patchy species distributions (Gibert et al., 1994). This habitat fragmentation leads to biodiversity hotspots through speciation by vicariance and niche diversification (Gibert & Deharveng, 2002; Dole-Olivier et al., 2009b; Malard et al., 2009; McInerney et al., 2014). At the small-scale (Fig. 1.2) short-range endemism in subterranean habitats is so high that some species are restricted to microhabitats, such as single drip pools in caves (Galassi et al., 2009b). Furthermore, the distribution of groundwater communities may track subterranean nutrient and oxygen concentrations, which vary greatly in space and time (Malard & Hervant, 1999; Datry et al., 2005).

Our knowledge of species distributions is best for karstic rocks (e.g. caves in the USA, Australia and Slovenia), which harbour the highest stygobite abundance and diversity (Christman et al., 2005; Humphreys, 2006; Culver & Pipan, 2009). One of the main reasons for this is that karstic rocks can be sampled directly in caves, whereas sampling in fractured and porous rocks relies on boreholes and wells. These are expensive and time-consuming to construct, and may be biased towards more permeable sections of the aquifer. A lack of suitable access points and their uneven distribution are reported as limiting factors in many studies (e.g. Christman et al., 2005; Eberhard et al., 2005). The

high prevalence of stygobites in cave systems has resulted in further sampling in similar habitats, as researchers have tried to maximise the chances of finding stygobites and recording endemics. In contrast, relatively few studies have sampled fractured habitats extensively (Dole-Olivier et al., 2009b; Hahn & Fuchs, 2009; Johns et al., 2015). However, despite lower abundances in fractured rocks, it is becoming increasingly evident that they harbour stygobites (Humphreys, 2006, 2008; Johns et al., 2015).

One example of this global trend of sampling bias can be seen in the UK. Here the most thorough sampling has been conducted in the Chalk (Fig. 1.3), a karstic habitat that is known to be a good habitat for stygobites in England (Proudlove et al., 2003; Robertson et al., 2009; Sorensen et al., 2013; Maurice et al., 2015). The Carboniferous Limestone, a karstic cave habitat, has also been sampled extensively due to easy access to the cave systems (Fig. 1.3). Meanwhile, other types of geology have been sampled much less (Fig. 1.3). For example, fractured rocks, such as different types of sandstone, cover large areas of the UK and Europe (see Cornu et al., 2013), but make up only a small proportion of the total sampling effort. Unconsolidated porous rocks have also not been sampled, although they harbour diverse groundwater communities in other geographic areas (Hahn & Matzke, 2005; Dole-Olivier et al., 2009a; Hahn & Fuchs, 2009; Malard et al., 2009).

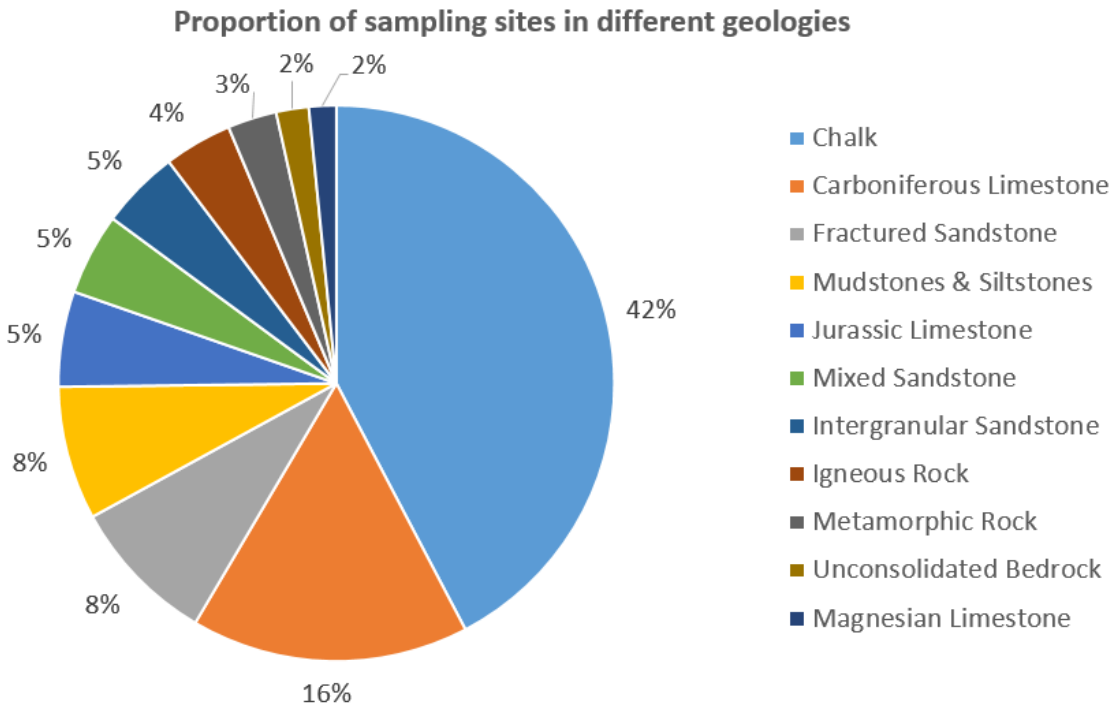


Fig. 1.3: Pie chart illustrating the proportion of groundwater samples taken in UK geologies, showing a strong sampling bias towards the Chalk and the Carboniferous Limestone. Other limestones, fractured and porous rocks have been sampled far less.

This sampling imbalance has recently been acknowledged (Dole-Olivier et al., 2009b; Hahn & Fuchs, 2009; Maurice & Bloomfield, 2012) and calls for additional research on fractured rocks have been put forward (Hahn & Fuchs, 2009; Larned, 2012). A recent study in south-west England addressed this by sampling a range of karstic, porous and fractured habitats in proportion to their geographical size (Johns et al., 2015). In the UK, several fractured and porous geologies covering substantial geographical areas have little or no sampling coverage, and very little is known about their importance to groundwater ecosystems. Understanding their ecology may also be important in a wider context, augmenting what little is known about similar habitat types elsewhere.

Some geo-habitats identified in chapter two had a much better sampling coverage (e.g. Chalk, Highly Karstic Limestone) than others (e.g. Fractured Sandstone, Mildly Karstic Limestone). Chapter three aimed to reduce the sampling bias present in England and Wales by taking samples in geologies that were not investigated previously (Devonian Sandstone, Lower Greensand) or that were insufficiently sampled (Jurassic Limestone, Magnesian Limestone). Devonian Old Red Sandstone, a fractured sandstone in south-east Wales, and Lower Greensand, a mixed fractured-porous sandstone in south England, cover relatively large areas (Jones et al., 2000) and occur within the distribution ranges of several stygobite species. If they provide suitable habitat, at least some species should occur within these geologies. Two limestone habitats, Magnesian Limestone (a north-south trending belt in northern England) and Jurassic Limestone (a belt further south), only have a sparse sampling coverage given their extensive outcrop areas. Because they are karstic they should sustain similarly diverse and abundant communities to those that have been found in karstic habitats elsewhere (Culver & Sket, 2000; Robertson et al., 2009; Maurice et al., 2015). Sampling was conducted in the northern Jurassic Limestone and the southern Magnesian Limestone to assess whether species have dispersed throughout the entire outcrops of these aquifers. Previously collected samples from the northern outcrop of the Magnesian Limestone north of the Devensian glacial limit did not contain stygobites (unpubl. data), possibly due to extirpations during the glaciation and the low dispersal capacity of groundwater organisms (Galassi, 2001; Rundle et al., 2002; Proudlove et al., 2003; Robertson et al., 2009). As the Magnesian Limestone occurs as a north-south trending outcrop which crosses the maximum southern limit of the Devensian ice sheet, sampling to the south of this limit was also undertaken to improve our understanding of the impact of the glaciation on stygobites.

Overall, sampling in these four geologies was conducted to assess their ecological communities and to identify whether they may be geologically controlled (mid-scale control, Fig. 1.2). Furthermore, it was investigated whether species have dispersed throughout the entire areas of geo-habitats and how species distributions relate to the Devensian glacial limit. Working towards more balanced datasets globally and nationally (i.e. undertaking sampling in poorly sampled geologies) and a better geographical coverage is a necessary prerequisite to carrying out broad distribution studies. The sampling effort in the four geologies intended to enhance our understanding of their ecology and to contribute data to the national-scale study that forms chapter four.

1.7 Groundwater controls on faunal biomass

An important consideration in assessing geological habitats and other environmental controls is the choice of response variables. Most studies investigating groundwater ecosystem controls focus on the presence-absence and abundances of species, and community structure (Hahn & Fuchs, 2009; Robertson et al., 2009; Stoch et al., 2009; Johns et al., 2015; Maurice et al., 2015). However, other parameters, such as biomass, may be equally suitable to assess controls on groundwater ecosystems. Biomass is the main energy currency driving ecosystems via the transfer of carbon through successive levels of the food chain (Reiss & Schmid-Araya, 2008, 2010). Estimating ecosystem biomass has a long-standing history in surface water studies (Fittkau & Klinge, 1973; Carpenter et al., 1985; Pace et al., 1999), but much less so in groundwater habitats. The heterotrophic nature of groundwater habitats means that nutrients, which are required to accumulate biomass, are generally scarce in the subterranean (Gibert et al., 1994).

Geology is known to control oxygen and nutrient concentrations at individual sites (small-scale control; Fig. 1.2), and is therefore also likely to affect the biomass of resident communities.

Although the focus in this thesis is on stygobites, obligate groundwater crustaceans, these animals tend to be rare in most northern European countries. For example, Johns et al. (2015) found stygobites at only 33.8 % of sites, and there were also insufficient stygobites in the four geologies to enable a meaningful analysis of the factors determining their biomass. Being stygophilic, copepods may provide a valuable alternative faunal target group for assessing biomass controls. Overall, copepods are important components of groundwater ecosystems and 6 of the 10 known copepod orders contain stygobites. 70 % of the species in the order Harpacticoida and 60 % of the species in the order Cyclopoida are stygobitic (Sket, 1999; Robertson et al., 2009). Copepods are also often much more abundant in groundwater communities than stygobite amphipods both within (Johns et al., 2015; Maurice et al., 2015) and outside (Galassi, 2001; Galassi et al., 2009b) the UK.

The environmental drivers influencing copepod communities have received considerable attention in recent decades. In hyporheic zones copepod communities are primarily determined by species-specific responses to habitat patchiness (Galassi et al., 2009). For example, in a French alluvial aquifer the species *Elaphoidella leruthi leruthi* was primarily determined by hydrogeological features (e.g. permeability), while *Parastenocaris meridionalis* occurred uniformly along the same gradient (Paran et al., 2005). In an Italian spring, copepod diversity varied with hydraulic conductivity and grain size composition at individual sites, with highest diversities being found in porous habitats

(Fiasca et al., 2005). Therefore, it has been established that copepods, like stygobites, are influenced by environmental parameters across a range of spatial scales (Fig. 1.2).

The latter part of chapter three addresses the issue that much less is known on the drivers of copepods in karstic rocks and other true groundwater habitats (Galassi et al., 2009), although it is increasingly clear that these are the habitats with the highest copepod diversity (Stoch, 1997; Pipan & Culver, 2005). Indeed, most groundwater studies report copepod abundances in bedrock (e.g. Johns et al., 2015; Maurice et al., 2016), but seldom investigate the controls of these abundances. Additionally, the influence of geology and other environmental parameters on copepod biomass in groundwater has not been investigated. Several abiotic parameters (e.g. distance to surface water and superficial deposit cover) related to oxygen and nutrient provision are good candidates for explaining biomass. For example, the extremely low DO and DOC concentrations in the Chalk confined by low-permeability clays of Quaternary or Palaeogene age (Shand et al., 2003) may limit copepod biomass and abundance. Equally, nutrient and oxygen concentrations may be lower with increasing distance to surface water and reduced hydrological connectivity (see Hahn, 2006; Bork et al., 2009; Culver & Pipan, 2009; Schmidt & Hahn, 2012). Chapter three estimates the total faunal biomass contained in sampling sites of the four geologies, by using published length-width regressions or geometric formulae. Furthermore, it investigates which environmental parameters best predict copepod biomass in the study area.

1.8 Geology and associated heterogeneity as stygobite controls

A recent cooperative project (PASCALIS) has elucidated groundwater biodiversity patterns across six European regions (see Gibert et al., 2009 for a project synthesis) and provided the first large-scale effort with a common, standardised sampling protocol and an equal focus on both karstic and non-karstic aquifers. One of the main conclusions of the PASCALIS study was that geology contributes significantly to ecosystem biodiversity with the most species-rich assemblages occurring in karstic aquifers and alluvial gravels (Dole-Olivier et al., 2009a; Galassi et al., 2009a), and depauperate communities occurring in fractured and compact aquifers (Hahn & Fuchs, 2009). Karstic and porous rocks appear to be similarly good habitats, as Martin et al. (2009) only found marginal differences between these aquifer types. Galassi et al. (2009a) also acknowledged the importance of habitat heterogeneity for stygobite assemblages, but stated that further research addressing this is needed.

In the UK only few stygobite distribution studies have been carried out (Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015) (Table 1.2), and these have been either restricted to a small number of geologies (Robertson et al., 2009; Maurice et al., 2015) or to a specific geographic area (Johns et al., 2015). Similar to studies outside the UK, these studies have identified some degree of geological control, detecting different stygobite communities in the various geologies. For example, stygobites were significantly less common in fractured habitats (e.g. mudstones and sandstones) than in carbonate aquifers (Johns et al., 2015; Maurice et al., 2015). Johns et al. (2015) were the first to sample fractured habitats in the UK and found that igneous and metamorphic rocks had much higher stygobite occurrences than mudstones and siltstones, which had the lowest

occurrence of any habitat. Although fractured rocks probably have a lower overall importance than karstic and porous rocks, accumulating evidence suggests future ecology sampling and analyses of faunal communities in fractured rocks are needed. To address this, two fractured geologies were sampled in chapter three, contributing new data to the national-scale distribution assessment in chapter four. The analysis undertaken also included hundreds of previously collected and unpublished stygobite records.

The habitat typology that was developed in chapter two of this thesis may provide a suitable framework for assessing stygobite distributions at the national scale, because it has high geological resolution and therefore incorporates more heterogeneity (mid-scale control, Fig. 1.2). Furthermore, the associated habitat quality scores incorporate hydrogeological variability (feeding into large-scale control, Fig. 1.2) and hydrochemical variability (feeding into small-scale control, Fig. 1.2). Providing the first national-scale survey, the habitat typology was used as a framework to analyse stygobite distribution data in chapter four. It was assessed whether more highly resolved geo-habitats harbour different communities and whether stygobite distributions are controlled by geology. The low biodiversity (8 species in England and Wales) offers the unique opportunity to study species distributions individually.

Understanding the distributions of stygobites at large-scale is important to assess ecosystem occurrence and plan conservation efforts. However, the functional importance of these ecosystems manifests at much smaller scales, such as individual aquifer fractures or boreholes (small-scale, Fig. 1.2). Occurrence of stygobites at this scale may have important implications for the functioning of ecosystems, which is addressed in the following section.

Table 1.2: Summary of UK studies that have investigated geological controls of stygobite assemblages.

Study	No. of habitats investigated	Scale	Mapping resolution	Stygobite patterns
Arietti & Edwards (2006)	1	Regional (833 km^2)	Not Indicated	Stygobites widespread (88 % of sampled sites)
Robertson et al., (2009)	6	National (England & Wales)	1:625k	Stygobites mainly in karstic rocks and south of glaciation
Johns & Dunscombe (2011)	16	Regional (Devon, Dorset, Durham)	1:625k	Stygobites mainly in karstic rocks and superficial deposits
Johns et al., (2015)	5	Regional (Devon & Dorset)	1:50k	Stygobites mainly in karstic and igneous rocks
Maurice et al., (2015)	1	Regional (Chalk aquifer)	Not Indicated	Stygobites widespread (67 %), but more common in south England
Weitowitz et al. (submitted) Current study	11	National (England & Wales; 151,156 km^2)	1:50k	To be determined

1.9 Surface water and groundwater food webs

Overall, the functioning of ecosystems is regulated through a series of complex, interdependent processes (Carpenter et al., 1985). Ecosystem services are the natural processes through which nature helps to sustain and fulfil human life (Daily, 1997) and, in groundwater, include bioremediation, the provision of clean drinking water, the maintenance of aquifer properties and flood control (Boulton, 2000; Herman et al., 2001; Danielopol et al., 2003). They are mostly provided by chemical reactions controlled by microbial activity, which are in turn regulated by interacting bottom-up and top-down forces. The bottom-up transfer of energy stored in nutrients allows primary consumers to grow and reproduce, increasing the biomass at these higher trophic levels (McQueen et al., 1989). Top-down control is the consumption of organismal carbon by consumers, grazers or predators, which typically reduce prey biomass (Sih et al., 1985). Although prey abundance is reduced, the metabolic activity and associated ecosystem services may be significantly enhanced by grazing (Wey et al., 2012).

Food web dynamics are well studied in aquatic surface ecosystems and both bottom-up and top-down processes contribute to its overall functioning (Elser et al., 2007; Maron & Crone, 2006). For example, high nutrient concentrations often increase microbial biomass and respiration (e.g. Craft et al., 2002), and this stimulatory nutrient effect is often propagated to higher trophic positions in the food chain (Posey et al., 1995). Top-down control typically reduces prey abundance and biomass (Adrian & Schneider-Olt, 1999; Reiss & Schmid-Araya, 2008, 2010), sometimes outweighing bottom-up nutrient effects (Rosemond et al., 2001). However, despite this regulatory function of lower trophic levels, grazing or predation may also enhance biofilm growth (Wey et al.,

2012) and ecosystem processes, such as the maintenance of aquifer functionality and contaminant degradation (Mattison et al., 2002, 2005).

In comparison to surface ecosystems, there are few experiments and field studies on groundwater food web dynamics (Larned, 2012). However, a better understanding of groundwater communities is the key to preserving them and the ecosystem services they provide. Although there is little empirical evidence of the grazing effects of stygobites, it is thought that they feed on both microbes and protozoans (Gibert et al., 1994; Hakenkamp & Palmer, 2000; Humphreys, 2000; Boulton et al., 2008), reducing prey abundance while possibly enhancing prey activity rates (Hancock et al., 2005). Because 99 % of subsurface bacteria are attached to a substrate (Lehman et al., 2001), stygobites most likely graze on attached biofilm or ingest sediment particles coated with bacteria (Hancock et al., 2005).

Typical groundwater food webs are truncated (Gibert & Deharveng, 2002) and mostly composed of microbes, protozoans and stygobite consumers (Fig. 1.4). Some field studies have found evidence for feeding relationships in groundwater ecosystems, as the abundance and diversity of stygobites significantly correlated with microbial abundances (e.g. Scarsbrook & Fenwick, 2003). In hyporheic zones, micro- or meiofauna (e.g. protozoans or copepods) act as carbon mediators that transfer energy from microbes to large crustacean invertebrates (Giere, 1993; Hakenkamp & Palmer, 2000; Mauclaire et al., 2000; Mermillod-Blondin et al., 2003) (Fig. 1.4), but these linkages remain understudied in true groundwater habitats.

Few experimental studies have investigated dynamics in groundwater food webs (e.g. Edler & Dodds, 1996; Kinsey et al., 2007; Cooney & Simon, 2009; Foulquier et al., 2010, 2011). Bottom-up nutrient control generally had a stimulatory effect (Cooney & Simon, 2009; Foulquier et al., 2010, but not Edler & Dodds, 1996), whereas evidence for a top-down stygobite grazing control remains inconclusive (e.g. Foulquier et al., 2010) (Fig. 1.4). Some studies found that microbes in biofilms grazed by stygobites had increased growth and activity rates (Edler & Dodds, 1996; Kinsey et al., 2007), while other researchers determined that grazing reduced bacterial activity (Cooney & Simon, 2009) or had no effect on bacterial parameters (Foulquier et al., 2011).

One of the reasons why grazing effects on bacteria in groundwater studies are difficult to demonstrate may be their rapid generation times, which enable them to respond quickly to disturbance (Findlay et al., 1990; Adrian & Schneider-Olt, 1999; Danovaro, 2000). Alternative response variables may allow easier detection of grazing effects. Protozoans (e.g. ciliates, flagellates and amoebae) are a diverse group of unicellular eukaryotic organisms that make up a significant proportion of the faunal abundance and biomass in aquatic ecosystems, yet are not considered in most studies (Kolasa, 2002; Stead et al., 2003; Reiss & Schmid-Araya, 2008, 2010). Protozoa may also control essential ecosystem services (e.g. denitrification and biodegradation of pollutants) by feeding on bacteria (Novarino et al., 1997; Strauss & Dodds, 1997).

While the regulatory link between protozoa and bacteria has been established (at least in the hyporheos), the trophic effects of stygobites are uncertain (Fig. 1.4). Their top-down control may be important in maintaining the aforementioned ecosystem services (Tomlinson & Boulton, 2008). Biodiversity loss is known to negatively affect

ecosystem services (Jonsson et al., 2001; Loreau et al., 2001) and the UK stygobite diversity is generally very low. This may increase the susceptibility of local ecosystems to species loss compared to regions that are hotspots of biodiversity, where species can be functionally replaced by others (e.g. Australia and Slovenia) (Walker, 1992; Boulton et al., 2008).

In the UK the Chalk is one of the best habitats, harbouring the highest biodiversity and abundance of stygobites in the UK (Johns et al., 2015; Maurice et al., 2015). Because of the high UK population density, pressure on groundwater resources is high and the Chalk is the most important aquifer in the UK providing almost 60 % of the total groundwater abstractions (Allen et al., 1997). The large voids that make the Chalk a good habitat also make it particularly vulnerable to anthropogenic pollution, to which stygobites are thought to be highly susceptible (Boulton et al., 2008). For example, the rising nitrate concentrations in Chalk groundwater are of increasing concern (Limbrick, 2003), because excessively high nitrate levels over 150 mg/l may result in the loss of groundwater taxa (Di Lorenzo & Galassi, 2013). Pollution incidents such as the catchment-scale bromate contamination of the Chalk in southern England (Cook et al., 2012) may also be a threat to groundwater ecosystems, although the effects of bromate on fauna are not known. The low biodiversity in the UK and increasing human pressure on groundwater habitats both in the UK and worldwide, make it particularly important to elucidate the role of stygobites in regulating groundwater ecosystem function, so that appropriate water management and conservation efforts can be planned.

The aim of the complementary experiments described in chapter five of this thesis was to mimic simple, but realistic groundwater food webs and to test factors that have regulatory function in other food webs. This included testing consumers (amphipods, isopods) with contrasting feeding strategy, as these may have different feeding efficiency (Robertson & Mann, 1980) and prey selectivity (Sommer, 1999). Furthermore, different consumer densities were tested, as grazing effects in some surface ecosystems have been shown to be density-dependent (Abrams & Ginzburg, 2000; Engkvist et al., 2000; Wright et al., 2005). These experiments should be set into the context of stygobite distributions, as these will determine geographically where in the UK top-down stygobite regulation may be most important.

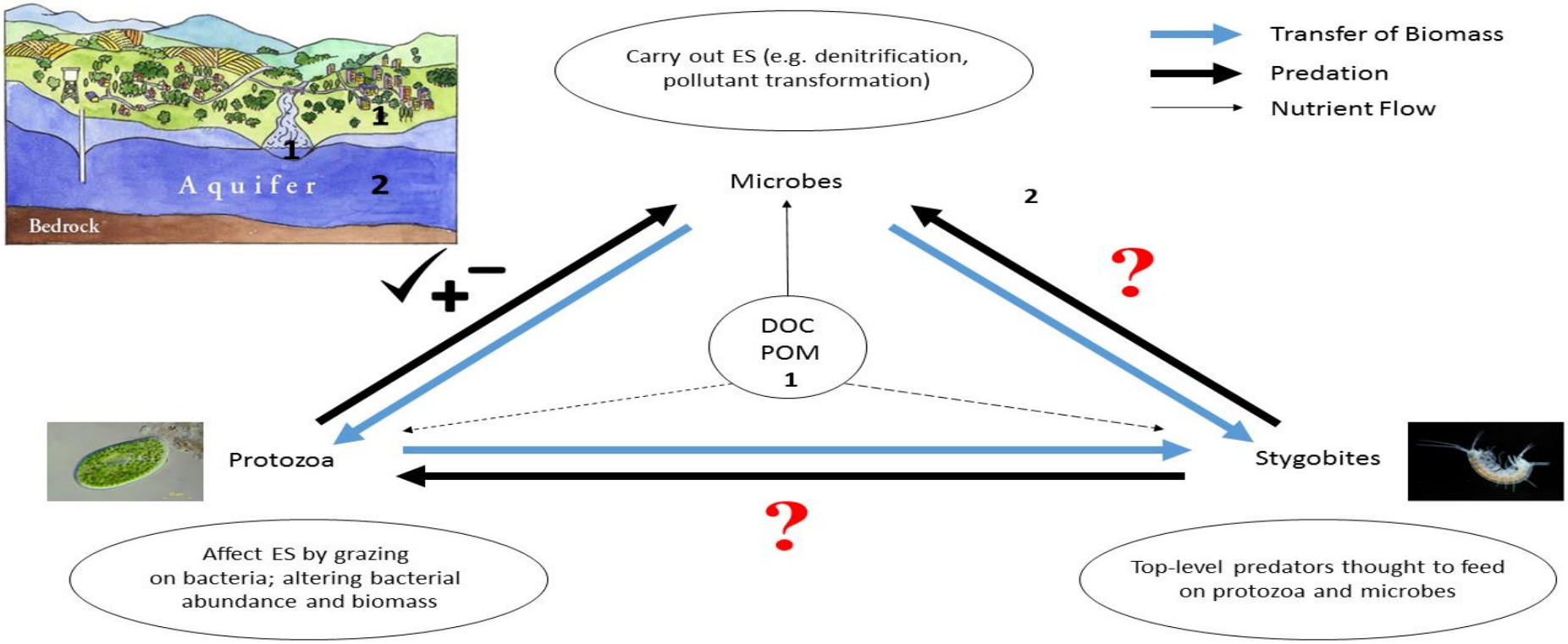


Fig. 1.4: Schematic diagram of the feeding relationships in truncated groundwater ecosystems, showing where organic matter originates (1) and where these food webs occur in aquifers (2). Thin black represent the flow of nutrients to bacteria and to a lesser extent to protozoans and stygobites (dashed lines). The thick black arrows represent feeding relationships, while the blue arrows represent the transfer of carbon between trophic levels. The trophic relationship between protozoa and microbes is documented, while little evidence exists for feeding effects of stygobites on either bacteria (lack of evidence) or protozoans (not studied). ES = ecosystem services, DOC = dissolved organic carbon, POM = particulate organic matter.

Chapter 2 – Defining geo-habitats for groundwater ecosystem assessments

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2.1 Abstract

Groundwater ecosystems comprising micro-organisms and metazoans provide an important contribution to global biodiversity. Groundwater organisms are known to be dependent on geology, which determines the physical habitat available, and the chemical conditions within it. Despite this, methods of classifying groundwater habitats using geological data are not well established and researchers have called for the incorporation of hydrogeological and hydrochemical data into habitat frameworks. Initially, 11 geo-habitats distinguished on hydrogeological principles were mapped. Hydrogeological (e.g. transmissivity) and hydrochemical (e.g. dissolved oxygen, carbon, nitrate, calcium) data were used to determine the characteristics of each geo-habitat, and demonstrate their differences. Furthermore, based on these characteristics a method to establish abiotic habitat quality to groundwater organisms was developed.

The hydrogeological grouping system provided geo-habitats with distinct properties relevant to groundwater ecosystems. As expected, karstic and porous habitats generally had a higher quality than fractured geo-habitats. All geo-habitats are highly heterogeneous, containing high-quality habitat patches that may harbour abundant and diverse groundwater communities. Extensive areas of England and Wales are covered by low-quality fractured habitats, highlighting the relative importance of high-quality habitats (e.g. Chalk) for groundwater biodiversity. These occur as north-south trending belts in central England, possibly facilitating faunal dispersal along a north-south axis. They are separated by outcrops of low-quality geo-habitats that may prevent the east-west dispersal of fauna. In south-western England and Wales high-quality geo-habitats occur as small isolated

patches. This study provides a national-scale typology that is adaptable for studies in other geographic areas.

2.2 Introduction

The earth's rocks and groundwater form unique, important habitats. Obligate groundwater invertebrates, and sometimes vertebrates, called stygobites are the top-level consumers in these truncated communities, feeding on a variety of organisms, including protozoans, microbes and fungi (Gibert et al., 1994; Boulton et al., 2008; Weitowitz et al., submitted). These groundwater communities are likely to be important due to their role in biogeochemical cycling and pollutant attenuation (Mattison et al., 2002, 2005). Stygobites also form a unique contribution to biodiversity because they are not found in other habitats, and have high rates of endemism and ancient ancestral lineages (Finston & Johnson, 2004; Lefebure et al., 2007; McInerney et al., 2014). Stygobite species diversity ranges from as little as eight in England and Wales to several hundred in countries such as Slovenia or Australia (Gibert et al., 1994; Eberhard et al., 2005; Robertson et al., 2009). Groundwater ecosystems depend on geology, which provides the physical habitat and affects the prevailing hydrochemical conditions (Datry et al., 2005; Hahn, 2006; Maurice & Bloomfield, 2012).

Generally, three types of physical structures are available as groundwater habitats: Pore spaces, fractures and karstic voids / caves. The quality of each of these types of habitat, i.e. their suitability to groundwater ecosystems in terms of species diversity and abundance supported, depends on the size and density of openings in the rock, as these, together with the nature of rock, determine space and ambient water

chemistry present in the subsurface. For example, habitat quality in unconsolidated sediments depends on pore space size, and is generally better in coarse-grained aquifers (Dole-Olivier et al., 2009; Hahn, 2009; Hahn & Fuchs, 2009; Malard et al., 2009). In fractured rocks habitat quality is highly variable and depends on fracture size, density and connectivity (Hahn & Matzke, 2005; Hahn & Fuchs, 2009; Maurice & Bloomfield, 2012). In contrast, karstic rocks are generally very good habitats, because dissolution has transformed fractures into large voids and cave systems with rapid water flow and surface connectivity (Danielopol et al., 2004; Malard et al., 2009; Robertson et al., 2009).

Grouping of habitats is frequently done for surface ecosystems and plays an essential role in ecology and conservation (Russ & Montgomery, 2002; Cañadas et al., 2005; Russo et al., 2005). For example, Cañadas et al. (2005) used physical and oceanographic variables important to cetaceans, in order to define different types of marine habitats. In groundwater studies, ranging in scale from the localised aquifer to the regional level, geologies have been mostly amalgamated into broad habitat categories (e.g. Castellarini et al., 2007; Dole-Olivier et al., 2009; Hahn & Fuchs, 2009; Robertson et al., 2009; Johns et al., 2015; summary of these studies in Appendix 2.1). These studies have determined that geology forms a major control on species distributions and abundances in groundwater ecosystems. While fractured rocks are generally characterised by communities of low diversity and abundance (Hahn & Fuchs, 2009), karstic and porous rocks have been found to harbour complex communities with high species abundances (Gibert et al., 2009; Robertson et al., 2009; Stoch et al., 2009). In a more recent approach, a groundwater habitat map based on the European hydrogeology map was developed, outlining the distribution patterns of different habitats on a continental scale (Cornu et al., 2013).

Aside from hydrogeological features, the quality of groundwater habitats depends on several chemical parameters. For example, it has been demonstrated that dissolved oxygen (DO), dissolved organic carbon (DOC), calcium and nitrate influence groundwater ecosystems, and the distribution of stygobites (Hahn, 2006; Dole-Olivier et al., 2009; Griebler et al., 2010). A study in the French Jura suggested that faunal distributions are primarily determined by DO gradients (Dole-Olivier et al., 2009), while another study found that DOC controlled the distribution of faunal communities (Datry et al., 2005; Hahn & Fuchs, 2009). Generally, rocks with higher permeability are thought to provide higher levels of oxygen and organic detritus than less permeable rocks (Hahn, 2006; Bork et al., 2009; Maurice & Bloomfield, 2012).

The need for more detailed typologies of groundwater habitats at the national and international scale, incorporating hydrogeological and hydrochemical data, has been highlighted in a recent review (Larned, 2012). Such typologies may aid in better assessing geological controls and predicting biodiversity, abundance and community structure (Castellarini et al., 2007; Tomlinson & Boulton, 2010; Stein et al., 2012). However, the relatively low resolution of current classification schemes may be limiting, as this is not representative of the true range of habitats available to groundwater ecosystems. Grouping many different geological strata into few units inevitably reduces the explanatory power of habitat frameworks, a problem which is exacerbated by the heterogeneity of rock (Stoch et al., 2009; Larned, 2012). A more detailed approach is necessary to assess geological controls on groundwater ecosystems and to identify species habitat preferences (Datry et al., 2005; Hancock et al., 2005; Tomlinson & Boulton, 2010). Ultimately, this will provide scientists with an improved tool for management and conservation decisions (Hahn, 2009).

The main aim of this study was to use detailed lithological and hydrogeological information to develop a geological habitat (geo-habitat) typology that is suitable for national-scale groundwater ecosystem studies. In contrast to previous studies, karstic, porous and fractured aquifers were further sub-divided to produce 11 distinct geo-habitats.

A secondary aim was to establish and compare the abiotic conditions (transmissivity, DO, DOC, nitrate and calcium) in the geo-habitats, and to develop a scoring system of habitat quality based on the mean and variability of these abiotic parameters. Furthermore, the distribution and connectivity of geo-habitats was assessed to determine where the most complex ecosystems with all trophic components (bacteria, protozoa, crustaceans) are expected to occur in England and Wales.

2.3 Methodology

In this study, 11 geo-habitats were conceptualised using hydrogeological principles. ArcGIS maps were developed in which geological units in England and Wales were assigned to the 11 geo-habitats. The hydrogeological and hydrochemical characteristics of the geo-habitats were investigated, and differences were determined, validating the classification. Finally, a habitat quality scoring system based on these characteristics was developed.

2.3.1 Assessing geo-habitat distribution

Determining geo-habitat categories

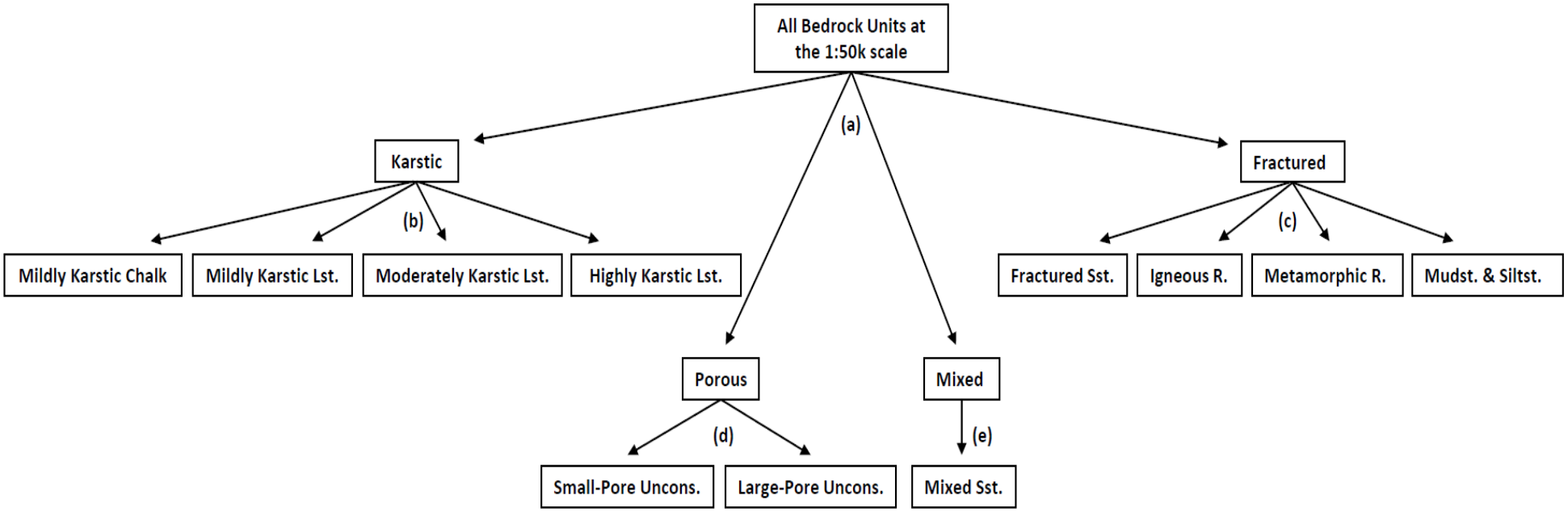

Fig. 2.1: The geo-habitat categorisation process. Lst. = Limestone, Sst. = Sandstone, R. = Rock, Mudst. & Siltst. = Mudstones & Siltstones, Uncons. = Unconsolidated

Initially all bedrock units mapped at the 1:50k scale in England and Wales were separated into karstic, porous and fractured rock, categories used in previous studies (e.g. Galassi et al., 2009; Hahn & Fuchs, 2009; Malard et al., 2009; Martin et al., 2009). Additionally, rocks with a mixture of intergranular and fracture water flow were assigned to a mixed rock category (Allen et al., 1997) (Fig. 2.1, step (a)). This mixed category formed one of the 11 geo-habitats that were defined. Both karstic and fractured rocks were further sub-divided into four categories, and porous rocks into two categories (Fig. 2.1).

Karstic aquifers were grouped into four geo-habitats based on previous classifications (Atkinson & Smart, 1981; Worthington & Ford, 2009) (Fig. 2.1, step (b)). These studies suggest that karstification increases from the Cretaceous Chalk, to the Permian Limestone, to the Jurassic Limestone and to the Carboniferous Limestone. Although caves are rare in the Chalk, solutional fissures and small conduits occur commonly (Maurice et al., 2006, 2012). The Permian Limestone is dolomitic and mildly karstic in nature. In the Jurassic Limestone caves are slightly more common, although the predominant habitat is solutional fissures and conduits. The Carboniferous Limestone provides both extensive cave systems (up to 100 km in length) and solutional fissures as habitat. The degree of karstification is important because it determines the habitat space and water chemistry available to groundwater ecosystems.

Fractured rocks were separated into four geo-habitats based on the size and density of fracturing (Jones et al., 2000) (Fig. 2.1, step (c)). Fractured Sandstone is a cemented rock with a relatively well developed fracture network, supporting moderate permeability (Jones et al., 2000). Igneous Rock and Metamorphic Rock both have low fracture densities, which is reflected in a low permeability (Jones et al., 2000). However,

as groundwater in Igneous Rock has higher oxygen, calcium, potassium and magnesium levels than Metamorphic Rock (Smedley & Allen, 2004; Shand et al., 2005), they were retained as separate geo-habitats. Mudstones & Siltstones are consolidated fine-grained rocks, which have very limited fracture networks with severely impeded water flow (Jones et al., 2000). Similar to the degree of karstification, fracture networks are important to groundwater ecosystems because they influence both the available habitat space and water chemistry.

Porous rocks were divided into Small-Pore Unconsolidated and Large-Pore Unconsolidated rock based on differences in grain and pore space size (Fig. 2.1, step (d)). Sediments with grain sizes below 2 mm (clays, silts, sands) were classified as Small-Pore Unconsolidated rock, while sediments with larger grain size were classified as Large-Pore Unconsolidated rock (e.g. gravels, flints, pebbles, boulders) (Jones et al., 2000). Post Quaternary superficial deposits also form porous habitats, but were not included in this study because there is insufficient information on the characteristics of these strata. Grain (and pore space) size is important because it determines habitat space and water chemistry in unconsolidated rocks.

Mixed Sandstone (mainly of Permo-Triassic age) is comprised of both cemented and unconsolidated sections, dominated by fracture and intergranular flow respectively (Allen et al., 1997) (Fig. 2.1, step (e)). Furthermore, these strata are characterised by a high variability in fracture size, fracture density, degree of cementation and mudstone content (Allen et al., 1997). The habitat quality in Mixed Sandstone is therefore likely to depend on locally dominant strata.

Assigning geological units to geo-habitats

Geological mapping of the UK is available at the 1:625k, 1:250k, 1:50k and 1:10k scales. The 1:50k geological map was used as it provides geological detail, accurate geological boundaries and a feasible number of geological units. ArcGIS 10.1 (ESRI, 2011), a geographic information system, was used to visualise the 10,000 different geological units in England and Wales. Attribute table information, the BGS online lexicon and the aquifer properties manuals (Allen et al., 1997; Jones et al., 2000) were consulted to assign units to the appropriate geo-habitats.

Units were first sorted by age, as this determines lithological features of rocks, such as the extent of karstification in carbonate rocks or consolidation in other sedimentary rocks (Worthington & Ford, 2009; Appendix 2.2). Because lithologies in the attribute tables (available from BGS; Smith et al., 2013) are sorted by dominance, a geological unit was incorporated into the geo-habitat that conformed to the dominant lithology.

For some geological units the categorisation was more complex. To decide whether sandstones were included in 'Mixed Sandstone' or 'Fractured Sandstone', information on age, consolidation and flow type was compiled from the BGS online lexicon (http://www.bgs.ac.uk/lexicon) and the England and Wales aquifer properties manuals (Allen et al., 1997; Jones et al., 2000). Sandstones of Carboniferous age and older are well cemented and were included in 'Fractured Sandstone'. Most Permo-Triassic sandstones have variable cementation and water flow and were included in 'Mixed Sandstone'. Only well cemented Permo-Triassic formations (e.g. Kidderminster Formation, Chester Formation) were classified as 'Fractured Sandstone'. Sandstones from

the Jurassic age and younger were included in ‘Mixed Sandstones’, because they contain areas with poor consolidation.

As part of the grouping process geological units with different characteristics sometimes had to be included in the same geo-habitat (Table 2.1). For example, ‘Fractured Sandstone’ contains the Devonian Old Red Sandstone, the Crackington Formation and the Millstone Grit, which have different degrees of mudstone impurities (Jones et al., 2000). ‘Small-Pore Unconsolidated’ includes sands, silts and muds with small pore spaces. ‘Igneous Rock’ is mostly comprised of granite, but also includes basalt, lava and tuff. The main geological units in ‘Metamorphic Rock’ are slate, gneiss, schist and quartzite. ‘Mudstones & Siltstones’ includes the Aylesbeare Mudstone, the Mercia Mudstone, the Raglan Mudstone Formation, coal and ironstone.

Table 2.1: Summary of the main lithologies and geological formations contained within each geo-habitat and their geological age range. Geological periods from which most units in a geo-habitat stem from are marked in **bold**.

Geo-habitat	Some of main lithologies and formations contained	Geological periods
Karstic Chalk	all chalk	**Cretaceous**
Mildly Karstic Limestone	oolite and Corallian limestones	**Jurassic** - Cretaceous
Moderately Karstic Limestone	limestone, dolostones	**Permian**
Highly Karstic Limestone	calcarenite, dolomitised limestone, dolostones	Neoproterozoic - **Carboniferous**
Small-Pore Unconsolidated	clay, sand, sand + clay, mud, silt	Cretaceous - **Quaternary**
Large-Pore Unconsolidated	gravel, sand + gravel	Cretaceous - **Quaternary**
Mixed Sandstone	Sherwood Sandstone Group, Kinnerton Sandstone Formation, Tunbridge Wells Formation	Cretaceous **–** **Permo-Triassic**
Fractured Sandstone	Old Red Sandstone, Crackington Formation, Millstone Grit, wacke	**Neoproterozoic** - Jurassic
Igneous Rock	andesite, basalt, gabbro, granite, lava, tuff	Neoproterozoic - Permian
Metamorphic Rock	gneiss, mylonite, quartzite, schist, slate	**Neoproterozoic** - Triassic
Mudstones & Siltstones	Aylesbeare Mudstone, Mercia Mudstone, coal, ironstone	Neoproterozoic - Cretaceous

2.3.2 Assessing geo-habitat characteristics

Data collection

Transmissivity and porosity data were obtained from the UK aquifer property manuals, including 1725 transmissivity values from pumping tests and 519 porosity values from core samples (see Table 2.2) (Allen et al., 1997; Jones et al., 2000). The distribution of sites with transmissivity data was uneven across geo-habitats (Weitowitz in prep., 2016), as pumping tests are often only performed on successful boreholes with relatively high yields. While the Chalk has the best coverage, Igneous Rock and Mildly Karstic Limestone are covered less comprehensively. Nevertheless, geographical coverage is good for all habitats except for the outcrops of Metamorphic Rock in northern Wales and Igneous Rock in northern England where no data are available. Porosity samples had less extensive coverage (Weitowitz in prep., 2016). Sampling coverage is evenly distributed for Mixed Sandstone and the Chalk, while for other geo-habitats such as Fractured Sandstone and Igneous Rock no data are available from south-western England and south-eastern Wales (Weitowitz in prep., 2016). The number of samples from Small-Pore and Large-Pore Unconsolidated rock is low.

Hydrochemical data, including DO, DOC, nitrate and calcium, were taken from the British Geological Survey (BGS) and Environment Agency (EA) Baseline Chemistry Report Series of aquifers in the UK (e.g. Ander et al., 2004; Cobbing et al., 2004; Smedley et al., 2004). Hydrochemical data from a faunal distribution study in south-western England were also used (Johns et al., 2015). In total 1412 DO samples, 998 DOC samples, 2342 nitrate samples and 2898 calcium samples were available (see Table 2.3). For several geo-habitats (e.g. the southern Chalk, Smedley et al., 2004; Moderately Karstic Limestone,

Griffiths et al., 2006) some data came from confined sites (i.e. with impermeable overlying strata), which typically have low oxygen and nutrient concentrations not representative of the rest of the aquifer. However, these were impossible to locate in the anonymised data set, which was therefore used in its entirety.

Comparing geo-habitat characteristics

Summary statistics of hydrogeological and hydrochemical variables were calculated in R (R Development Core Team, 2016). To determine whether data were normally distributed, histograms, q-q plots and Shapiro-Wilk normality tests were conducted.

Following Gagic et al. (2016), a principal component analysis was conducted in the R package 'missMDA' (Josse & Husson, 2016) to assess abiotic characteristics in broad habitat groups (karstic, porous, fractured). This package handles missing data by using a regularised mean substitute method, which takes the parameter mean and correlations between variables into account (Josse & Husson, 2012). As the PCA was conducted on mixed-type data, categorical variables were transformed into a disjunctive data table, before being scaled to unit variance using MCA scaling (Josse & Husson, 2012).

As all of the parameters did not follow normality, non-parametric Kruskal-Wallis tests were used to test for significant differences between geo-habitats. When these were significant ($P < 0.05$), post-hoc multiple pairwise comparisons with Bonferroni corrections were performed in the R 'Psych' package (Revelle, 2016) to determine which geo-habitats differed. To reduce the number of comparisons, the geo-habitat with the lowest median transmissivity was used as a point of comparison because this was expected to be the least suitable habitat and low transmissivity is likely to limit hydrochemistry.

2.3.3 Evaluation of geo-habitat quality

To assess the quality of geo-habitats, seven parameters known to influence groundwater communities were considered. These were dissolved oxygen (DO, Gibert et al., 1994; Dole-Olivier et al., 2009), dissolved organic carbon (DOC, Datry et al., 2005; Hahn, 2006), nitrate (NO_3^- , Stein et al., 2010), calcium (Ca, Rukke, 2002), transmissivity (permeability) (Hahn, 2006; Bork et al., 2009), cave development (Culver & Sket, 2000) and pore space size (Dole-Olivier et al., 2009). A method was developed to determine overall habitat suitability as well as habitat consistency, by identifying the number of suitable and unsuitable patches within geo-habitats. To do this, critical parameter thresholds (below which ecosystems would be adversely affected) were set, which were extracted from the literature. These threshold parameters were used as cut-off points to identify the ratio of good – bad quality patches.

Identification of thresholds and additional parameters

A critical threshold of 1 mg/l was set for DO, as previous studies found this concentration to be the lower critical survival limit of groundwater invertebrates (Malard & Hervant, 1999; Hahn, 2006). For DOC, the main food source in groundwater, a critical threshold of 0.4 mg/l was used, because this was the concentration below which taxa were lost from groundwater communities (Datry et al., 2005). For nitrate, an important additional resource for groundwater bacteria (Stein et al., 2010), the threshold was also set to 0.4 mg/l, because no previous study has indicated minimum nitrate requirements for groundwater bacteria. Calcium was set to a critical limit of 5 mg/l, because this was the minimum concentration needed for surface freshwater invertebrates to maintain their carapace (Rukke, 2002). For transmissivity a lower threshold of 51.7 m^2/d was set, which

was the average transmissivity in Mudstones & Siltstones (Jones et al., 2000), because compact aquifers typically support depauperate communities (Hahn & Fuchs, 2009; Johns et al., 2015). Geologies with lower transmissivities than this threshold are therefore likely to be unsuitable for groundwater communities.

Two additional parameters were incorporated into the scores (see calculation (b) below). Cave development was considered, as caves are known to provide particularly good groundwater habitats (Culver & Sket, 2000; Robertson et al., 2009). The extent of karstification differs among UK limestones (Atkinson & Smart, 1981; Worthington & Ford, 2009) with probable effects on groundwater ecosystems. Physical habitat space was considered because this is known to limit faunal distributions. For example, small pore spaces are known to limit groundwater assemblages by excluding larger invertebrates (Dole-Olivier et al., 2009).

Calculation

(a) The ratio of the number of sites above threshold / below threshold (+1 to avoid division by zero) was calculated for all parameters in each geo-habitat. The mean of each parameter was also ranked between all geo-habitats from 1 (lowest mean) to 10 (highest mean). The threshold ratio for each parameter was multiplied with the rank of its mean to give a habitat score for each parameter. Values for all parameters were then summed up to give an intermediate geo-habitat score (*IS*, see *eqn 1*).

(b) Each geo-habitat received a cave score between 1 (no caves) and 4 (extensive caves). Furthermore, geo-habitats either received a penalty score of 1 (physical space not limiting) or 2 (physical space excluding larger fauna). The IS of each geo-habitat was multiplied with the cave score and divided by the space penalty to give the final geo-habitat quality score (*FS, see eqn 2*).

The intermediate geo-habitat score (*IS*) was calculated as:

$$IS = \sum_{i=1,2,3,4,5} \left(\frac{A}{B+1} * Rm \right) \quad (1)$$

, where A is the number of sites within the same geo-habitat category above threshold of parameter *i*, *B* is the number of sites below threshold of parameter *i* and *Rm* is the rank of the mean of parameter *i*.

The final geo-habitat quality score (*FS*) was calculated as follows:

$$FS = \frac{IS * CS}{SP} \quad (2)$$

, where CS is the cave score (i.e. a value of 1, 2, 3 or 4) and SP is the space penalty (i.e. a value of 1 or 2).

2.4 Results

2.4.1 Distribution of geo-habitats

The distribution of geo-habitats, as defined here by distinguishing 11 categories (Fig. 2.1), is uneven across England and Wales (Fig. 2.2). Out of the 11 geo-habitats, three (Mudstones & Siltstones, Fractured Sandstone and the karstic Chalk) clearly dominate groundwater habitats in England and Wales, having an area coverage of 40.09 %, 18.21 % and 12.6 % respectively. The karstic Chalk forms a continuous band in south-eastern England, running from north to south (Fig. 2.2). Conversely, Mudstones & Siltstones and Fractured Sandstone have much more dispersed and patchy distributions (Fig. 2.2). Overall, the total coverage of broad habitat groups varies greatly. Fractured rocks are the most common type of groundwater habitat (63.15 %) in England and Wales, whereas karstic (18.54 %) and porous / mixed habitats (18.31 %) cover much smaller areas (Appendix 2.3).

The Mildly and Moderately Karstic Limestone, are also prevalent across extensive areas of central, southern and eastern England. They form continuous belts running on a north-south axis. Such belts are absent from Wales and south-western England, where Highly Karstic Limestone occurs in isolated patches.

Porous and mixed geo-habitats are almost entirely restricted to England, and generally have very patchy and disconnected distributions (Large-Pore Unconsolidated, Mixed Sandstone); with the exception of the Small-Pore Unconsolidated rock in eastern England (Fig. 2.2).

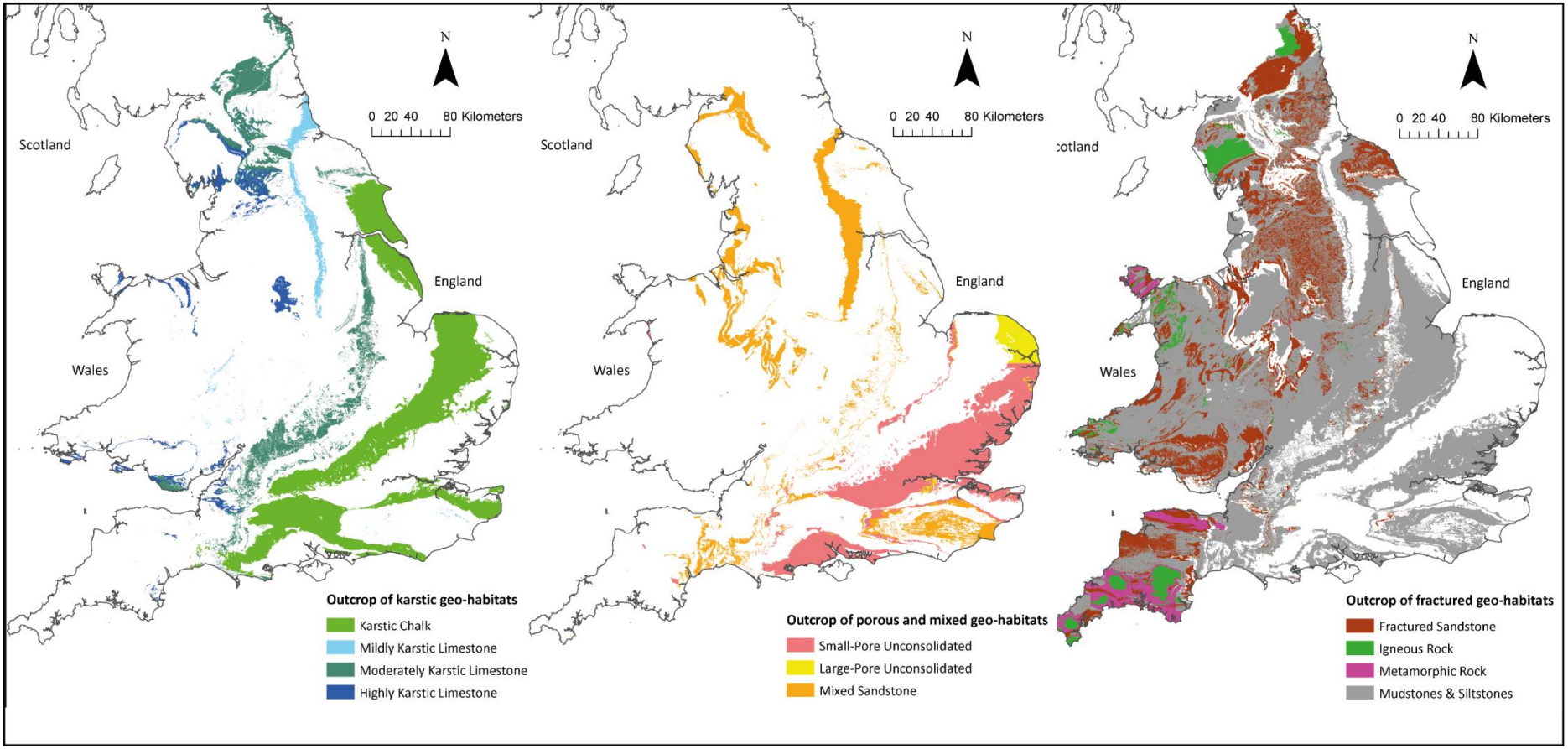

Fig. 2.2: Distribution maps of the outcrops of groundwater geo-habitats in England and Wales.

Fractured habitats occur widely in Wales, south-western, central and north-western England (Fig. 2.2). All fractured habitats are relatively disjointed and patchy, but greatly differ in the extent of their distribution. Igneous and Metamorphic Rock are relatively uncommon habitats limited to Wales, south-western and northern England. Fractured Sandstone and Mudstones & Siltstones are very widespread, covering extensive areas of England and Wales.

While some geo-habitats cover extensive areas, others such as Highly Karstic Limestone (2.06 %), Large-Pore Unconsolidated rock (0.98 %) and Mildly Karstic Limestone (0.96 %) only cover small geographic areas (Appendix 2.3). Overall, the geo-habitats in England and Wales range from frequent and well connected, to rare and isolated.

2.4.2 Geo-habitat characteristics

The PCA ordination indicated that geology is strongly associated with transmissivity (T) (Fig. 2.3a; summary statistics in Appendix 2.4), with fractured geo-habitats being characterised by low T, porous geo-habitats by intermediate T and karstic geo-habitats by high T (Fig. 2.3b). Geo-habitats are clearly distinguishable according to broad habitat type (karstic, porous and fractured) on the PCA biplot, with ellipsoids indicating a marginal difference in abiotic conditions between karstic and porous geo-habitats, while showing significant differences for fractured geo-habitats (Fig. 2.3c).

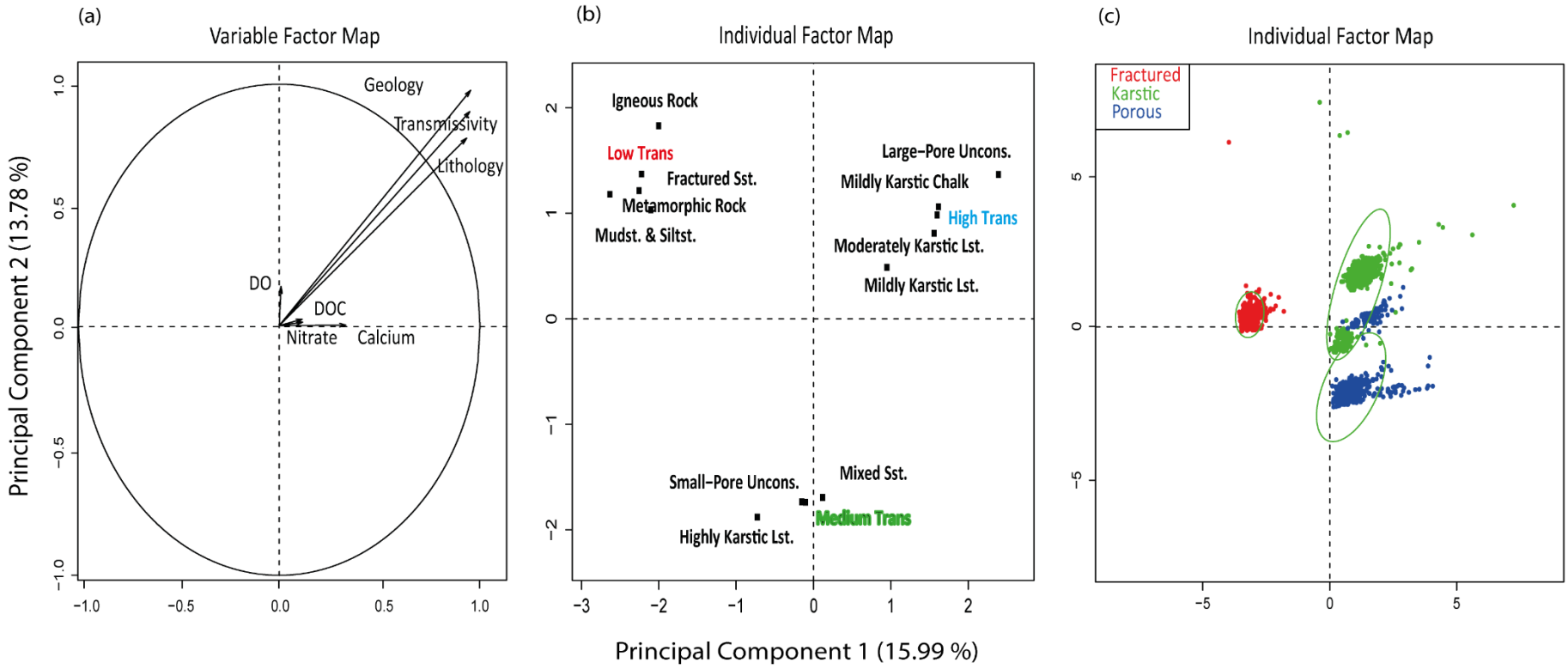

Fig. 2.3: (a) Ordination of environmental variables in a Principal Component Analysis (PCA), explaining a total of 29.77 % of data variance. (b) Ordination of geo-habitats grouped by different colour-coded transmissivities (low, medium, high). (c) Ordination of broad lithologies (fractured, karstic, porous habitats) with individual colour-coding. Confidence ellipsoids around broad groups indicate significant abiotic differences.

The first principal component axis (15.99 % explained variance) indicated that karstic and porous geo-habitats positively relate to transmissivity, DOC, nitrate and calcium, while fractured geo-habitats have a negative relationship with these parameters (Fig. 2.3). Highly Karstic Limestone was separated from the other karstic geo-habitats and appeared to be negatively associated with these parameters.

The second principal component axis (13.78 % explained variance) was characterised by a positive loading of DO (Fig. 2.3a). Karstic and fractured geo-habitats associated positively with DO concentrations, while porous geo-habitats were characterised by lower DO concentrations (Fig. 2.3). Again, Highly Karstic Limestone was separated from other karstic geo-habitats and characterised by a lower DO concentration. Overall, the PCA confirmed that a broad typology yields distinguishable broad karstic, porous and fractured groups, but that some geo-habitats clearly do not follow these general trends.

Hydrogeology

The geo-habitats had significantly different transmissivity (Kruskal-Wallis test: $H(10) = 799.58$, $P < 0.001$) (Fig. 2.4). The highest mean transmissivities occurred in karstic geo-habitats, such as Moderately Karstic Limestone and the Chalk (1504 m^2/d) (Fig. 2.4, Table 2.2). Medium transmissivities were found in Mildly Karstic Limestone, Large Pore Unconsolidated and Mixed Sandstone. The lowest transmissivities occurred in two fractured geo-habitats: Igneous Rock (13 m^2/d) and Metamorphic Rock (16 m^2/d). Highly Karstic Limestone had a similar transmissivity to Small-Pore Unconsolidated rock.

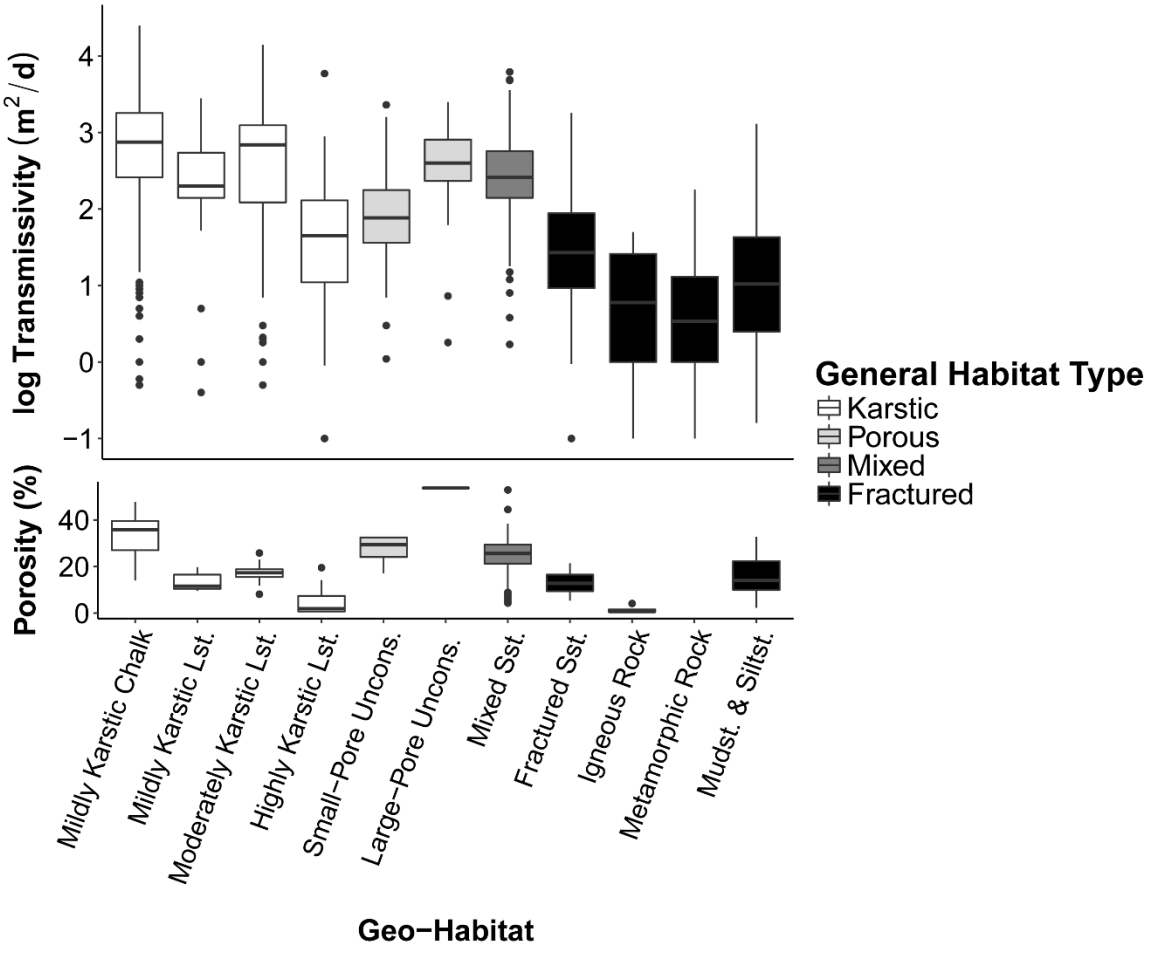

Fig. 2.4: Hydrogeological parameters of the 11 geo-habitats showing (a) log transmissivity (m^2/d) and (b) porosity (%). No porosity data was available for Metamorphic Rock. Lst. = Limestone, Sst. = Sandstone, Mudst. & Siltst. = Mudstones & Siltstones

Table 2.2: Number of samples, mean values, standard errors, minimum and maximum values of transmissivity and porosity for each of the geo-habitats. Arrows indicate significantly higher ↑ or lower ↓ levels of a parameter than the 'control' geo-habitat Igneous Rock according to multiple pairwise comparisons (Bonferroni corrected).

Geo-Habitat	Transmissivity (m^2/d)				Porosity (%)			
	Number of Samples	Mean (se)	Min	Max	Number of Samples	Mean (se)	Min	Max
Chalk	734	1504.2 (91.1) ↑	0.5	25000	80	33.8 (0.9) ↑	14	47.8
Mildly Karstic Limestone	22	502.7 (160.6) ↑	0.4	2800	5	13.5 (2)	9.5	19.7
Moderately Karstic Limestone	82	1628.8 (317.6) ↑	0.5	14000	25	17.1 (0.7)	8.1	25.8
Highly Karstic Limestone	33	317.4 (178.7) ↑	0.1	5900	15	4.9 (1.6)	0.3	19.5
Small-Pore Unconsolidated	40	252.1 (85.4) ↑	1.1	2300	4	27.2 (3.7) ↑	17.1	32.8
Large-Pore Unconsolidated	86	581.5 (53) ↑	1.8	2500	1	53.7 (NA) ↑	53.7	53.7
Mixed Sandstone	320	505.6 (41.7) ↑	1.7	6200	328	24.5 (0.4) ↑	4.3	52.9
Fractured Sandstone	147	111.9 (21) ↑	0.1	1800	21	13.2 (1.1)	5.3	21.4
Igneous Rock	13	13.4 (4.7)	0.1	50	4	1.5 (0.9)	0.4	4.1
Metamorphic Rock	71	16.1 (3.9)	0.1	180	0	NA	NA	NA
Mudstones & Siltstones	176	51.7 (9.2)	0.2	1300	35	16.1 (1.4)	2.3	32.8

Minimum transmissivities were similar between geo-habitats, ranging between 0.10 and 1.8 m^2/d. Maximum transmissivities varied between 50 and 25,000 m^2/d (Table 2.2). The transmissivity maxima were much lower in fractured geo-habitats than in karstic and porous geo-habitats. Transmissivity was highly variable in all geo-habitats, being most variable in karstic and porous habitats and less variable in fractured rocks (Fig. 2.4). For some habitats, such as the Chalk, transmissivity varied over several orders of magnitude (between 1 and 25,000 m^2/d) (Table 2.2). Overall, the transmissivity in fractured geo-habitats was always low, while karstic and porous habitats had many highly transmissive sites.

Porosity was also significantly different between geo-habitats ($H(10) = 206.5$, $P < 0.001$) (Fig. 2.4). In unconsolidated habitats (Small-Pore and Large-Pore Unconsolidated rock), mean porosity was high (> 24 %). In contrast, consolidated geo-habitats (except the Chalk; 33.8 %) had much lower porosity (< 17 %). The lowest recorded porosity (1.45 %) occurred in Igneous Rock (Fig. 2.4, Table 2.2).

Hydrochemistry

DO concentrations significantly differed between geo-habitats ($H(10) = 210.91$, $P < 0.001$) (Fig. 2.5). The highest mean DO concentrations occurred in the Chalk and Igneous Rock, while the lowest occurred in Mildly Karstic Limestone and Small-Pore Unconsolidated rock. Most porous geo-habitats had lower DO than fractured geo-habitats (Fig. 2.5, Table 2.3). All geo-habitats had sites with minimum DO concentrations varying between 0.02 and 0.48 mg/l (data not shown). Maximum DO concentrations varied between 9.23 and 104.3 mg/l, with the highest maxima being recorded in the Chalk and Igneous Rock.

Dissolved Organic Carbon (DOC) concentrations significantly differed between habitats ($H(10) = 73.04$, $P < 0.001$) (Fig. 2.5). The highest mean DOC was found in the Chalk and Moderately Karstic Limestone, two karstic geo-habitats, and in Mudstones & Siltstones (Fig. 2.5, Table 2.3). Intermediate DOC concentrations were found in porous and mixed geo-habitats, such as Mixed Sandstone, and lowest DOC concentrations occurred in fractured geo-habitats, such as Igneous Rock and Metamorphic Rock (Fig. 2.5). Minimum DOC showed relatively little variation between geo-habitats, ranging from 0.09 to 0.82 mg/l (data not shown). The maximum DOC concentrations of 292 and 207 mg/l occurred in the Chalk and Moderately Karstic Limestone.

Nitrate concentrations were significantly different between geo-habitats ($H(10) = 410.37$, $P < 0.001$) (Fig. 2.5). The highest mean nitrate concentrations occurred in the Chalk and Moderately Karstic Limestone, two karstic geo-habitats, and Large-Pore Unconsolidated rock (Fig. 2.5, Table 2.3). The lowest nitrate concentrations occurred in fractured geo-habitats, such as Fractured Sandstone and Metamorphic Rock (Fig. 2.5). Minimum nitrate concentrations in habitats were very low, ranging from 0.002 to 0.14 mg/l (data not shown). Maximum nitrate concentrations varied considerably between 12.7 to 72 mg/l, with the highest maxima in Mixed Sandstone, Small-Pore Unconsolidated and Highly Karstic Limestone.

Calcium also varied significantly between geo-habitats ($H(10) = 1260.64$, $P < 0.001$) (Fig. 2.5). The highest mean calcium concentrations occurred in the Chalk, Moderately Karstic Limestone and Large-Pore Unconsolidated rock, while the lowest calcium concentrations occurred in fractured geo-habitats, such as Igneous Rock and Metamorphic Rock (Fig. 2.5, Table 2.3). Despite a few outliers, minimum calcium was

fairly consistent between geo-habitats, ranging from 0.48 to 14.18 mg/l (data not shown). The maximum calcium concentrations ranged from 66.2 to 795 mg/l, with the highest maxima being found in Mixed Sandstone and Mildly Karstic Limestone.

2.4.3 Geo-habitat quality

The scores, which account for variability in habitat space and water chemistry, illustrated that habitat quality is considerably different between geo-habitats (Fig. 2.6). The highest quality score was recorded for the Chalk, which was three times higher than that of Metamorphic Rock, the habitat with lowest quality (see Appendix 2.5 for quality scores). For discussion purposes, geo-habitats were assigned to three broad groups with high (> 3.5), intermediate (2.5 – 3.5) and low (< 2.5) quality. The high-quality group includes the Chalk and Moderately Karstic Limestone, two karstic geo-habitats (Fig. 2.6). The intermediate-quality group includes two karstic (Mildly and Highly Karstic Limestone), one porous (Large-Pore Unconsolidated), one mixed (Mixed Sandstone) and one fractured (Igneous Rock) geo-habitat (Fig. 2.6). The low-quality group is comprised of one porous geo-habitat (Small-Pore Unconsolidated) and the remaining fractured geo-habitats (Fractured Sandstone, Metamorphic Rock, Mudstones & Siltstones) (Fig. 2.6). Given the importance of the included abiotic parameters to groundwater ecosystems, geo-habitat quality is expected to reflect differences in resident community complexity and abundance. Low-quality geo-habitats may harbour lower species diversity and abundance of all trophic levels (bacteria, protozoa, crustacea) than high-quality geo-habitats.

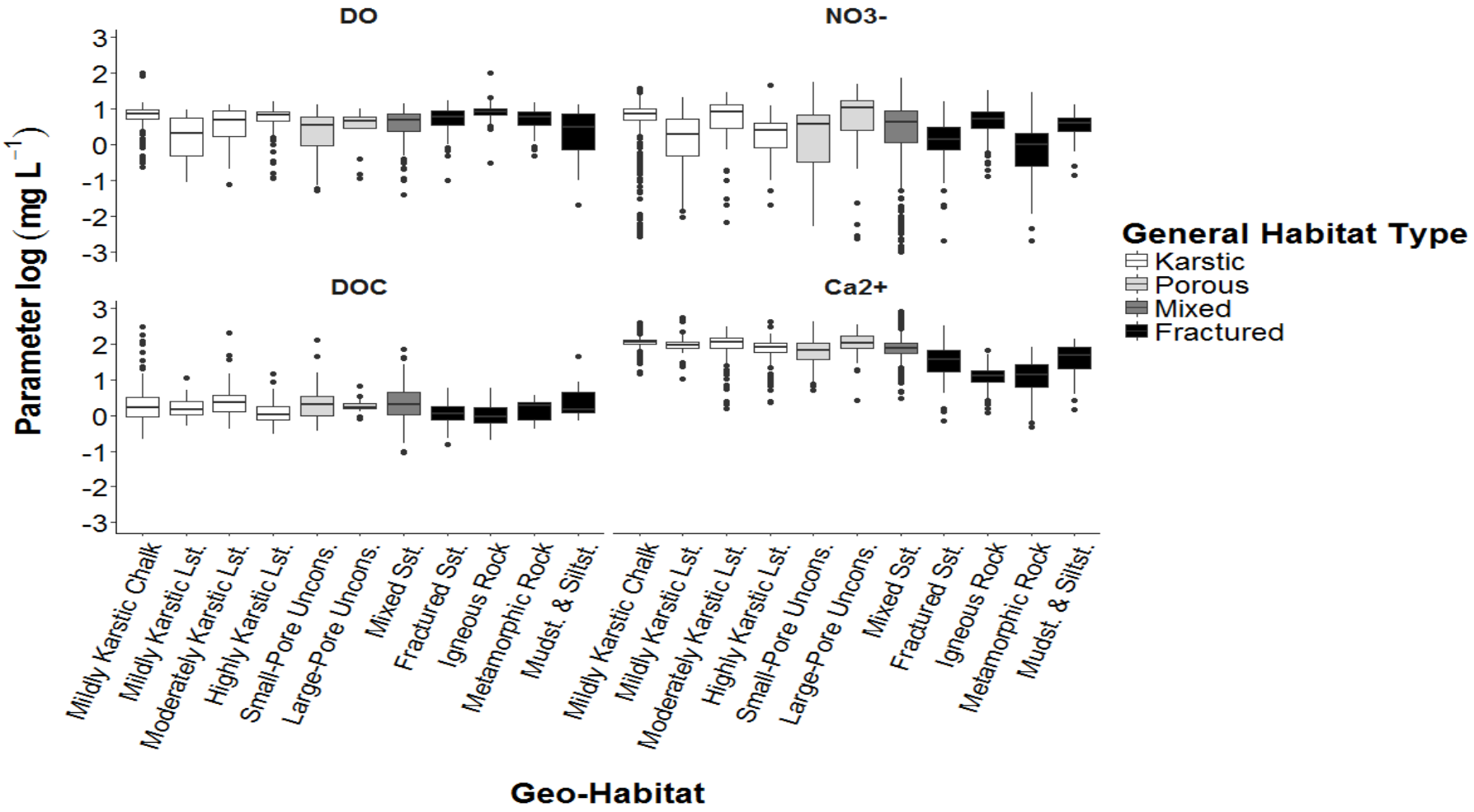

Fig. 2.5: Hydrochemical parameters of the 11 geo-habitats showing logs of dissolved oxygen (DO), dissolved organic carbon (DOC), calcium and nitrate (all in mg/l). Lst. = Limestone, Uncons. = Unconsolidated, Sst. = Sandstone, Mudst. &Siltst. = Mudstones and Siltstones

Table 2.3: Number of samples, the mean concentrations and standard errors of dissolved oxygen (DO), dissolved organic carbon (DOC), nitrate and calcium for each of the geo-habitats. Arrows indicate significantly higher ↑ or lower ↓ levels of a parameter than the 'control' geo-habitat Metamorphic Rock according to multiple pairwise comparisons (Bonferroni corrected).

Geo-Habitat	Number of Samples	Transmissivity (m^2/d) (se)	Number of Samples	DO (mg/l) (se)	Number of Samples	DOC (mg/l) (se)	Number of Samples	Nitrate (mg/l) (se)	Number of Samples	Calcium (mg/l) (se)
Mildly Karstic Chalk	734	1504.2 (91.1)	305	7.8 (0.5)↑	247	6.3 (1.7)	623	7.7 (0.2) ↑	680	110.5 (1.4)↑
Mildly Karstic Limestone	22	502.7 (160.6)	36	3.2 (0.5)↓	36	2.1 (0.3)	105	3.6 (0.4)	112	107.7(7.1) ↑
Moderately Karstic Limestone	82	1628.8 (317.6)	108	5.1 (0.3)	89	6.3 (2.4)	104	8.6 (0.6) ↑	171	107.7 (3.9)↑
Highly Karstic Limestone	33	317.4 (178.7)	103	6.5 (0.3)	98	1.7 (0.2)	181	3 (0.3)	229	80.4 (2.9) ↑
Small-Pore Unconsolidated	40	252.1 (85.4)	130	3.8 (0.7)↓	109	4.3 (1.3)	121	6.2 (0.9) ↑	209	76.3 (3.9) ↑
Large-Pore Unconsolidated	86	581.5 (53)	13	4.3 (0.8)	21	2.1 (0.3)	67	11.6 (1.3)↑	85	120.1 (7.3)↑
Mixed Sandstone	320	505.6 (41.7)	349	4.9 (0.2)	261	4.2 (0.4)	592	6.2 (0.3) ↑	708	100.5 (3.9)↑
Fractured Sandstone	147	111.9 (21)	124	6.2 (0.3)	79	1.5 (0.1)	204	2.6 (0.2)	282	44.6 2.2) ↑
Igneous Rock	13	13.4 (4.7)	96	9.6 (1) ↑	39	1.4 (0.2)	209	6.3 (0.3) ↑	213	14.9 (0.7)
Metamorphic Rock	71	16.1 (3.9)	97	5.9 (0.3)	5	1.8 (0.6)	98	2.2 (0.4)	122	18 (1.5)
Mudstones & Siltstones	176	51.7 (9.2)	51	4.2 (0.5)↓	14	5.8 (3.1)	38	4.6 (0.6) ↑	87	51.8 (3.7) ↑

The distribution map showing habitats grouped according to their quality, illustrates that most of England and Wales is covered by low-quality habitats, whereas medium- and high-quality habitats cover much smaller areas (Fig. 2.7). High-quality, and some medium-quality habitats, provide highly permeable corridors connecting southern and northern England, whereas most medium-quality habitats in Wales and southern England cover small geographical areas and are spatially patchy (Fig. 2.7). Low-quality habitats are dominant in much of the study area, particularly in Wales and south-western England, providing low permeability in extensive geographical areas. High-quality habitats are concentrated in central, eastern and southern England (Fig. 2.7), which is where the most complex ecosystems would be expected.

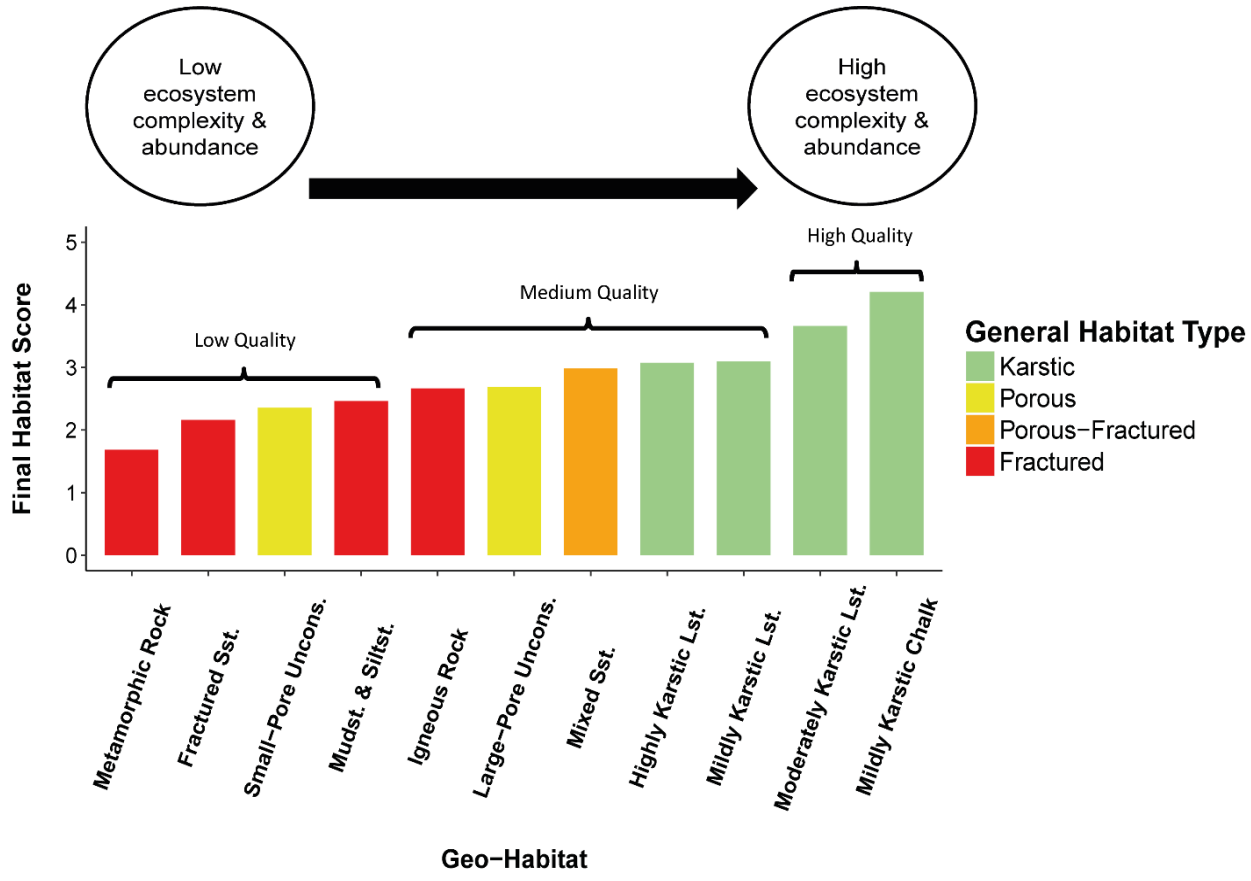

Fig. 2.6: Geo-habitat quality scores (log scale) ordered from lowest to highest and their potential implications for resident communities.

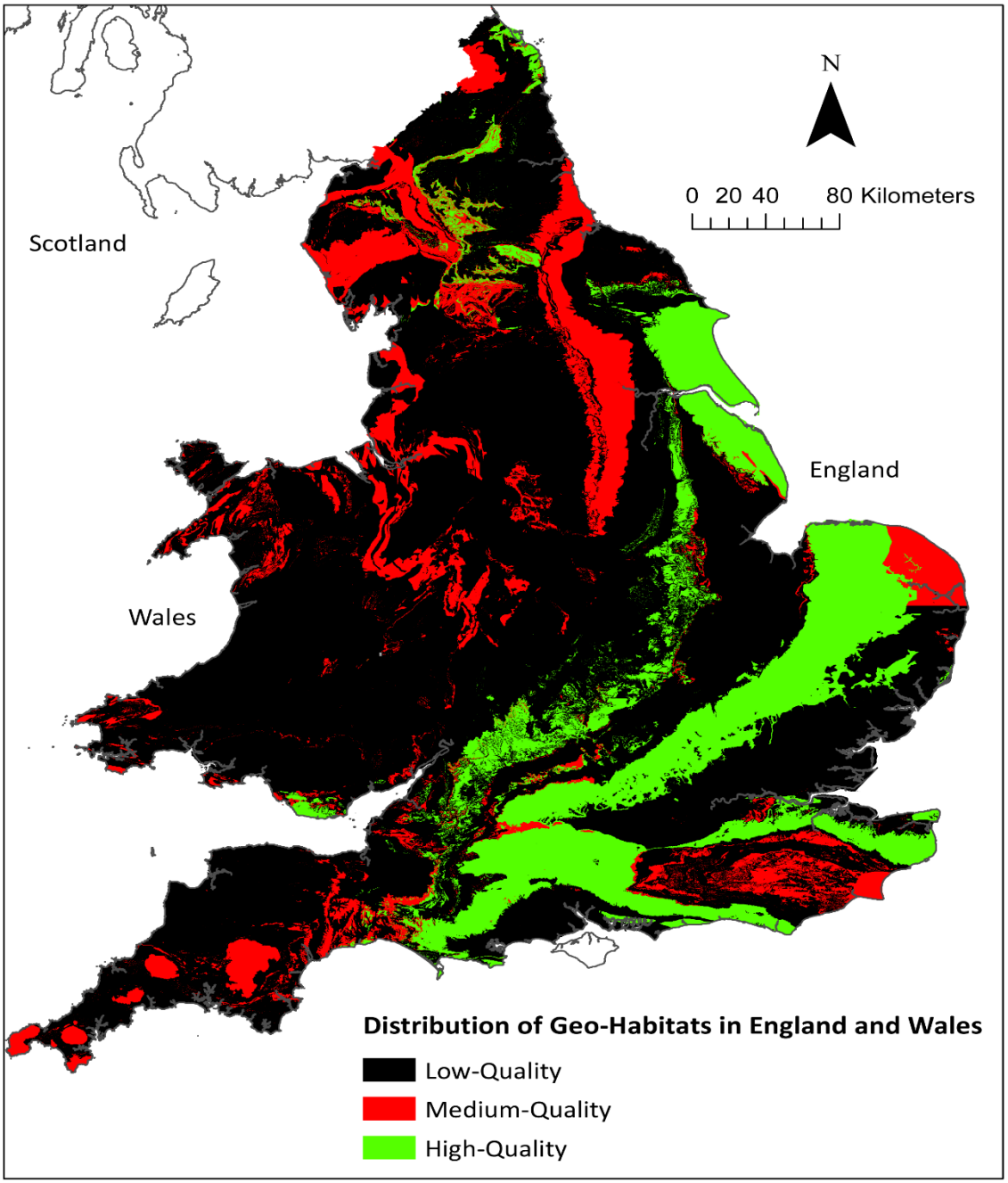

Fig. 2.7: Distribution of geo-habitats in England and Wales grouped according to their habitat quality scores calculated from abiotic parameters important to groundwater ecosystems.

2.5 Discussion

2.5.1 Assessing the new typology

Habitat grouping methods in groundwater studies have been relatively broad and calls for new typologies incorporating hydrochemical data have emerged (Larned, 2012; Cornu et al., 2013). In this study, the classical approach of dividing habitats into karstic, porous and fractured rocks was refined to include abiotic characteristics that are likely to be relevant to organisms. Detailed hydrogeological knowledge was used to define 11 geo-habitats, providing a higher resolution typology than those that have been used in previous groundwater studies.

Karstic, porous and fractured rocks broadly differ in hydrogeological and hydrochemical characteristics. As predicted, most karstic and porous geo-habitats were characterised by high transmissivities and suitable hydrochemistry, both prerequisites for supporting the abundant and diverse groundwater ecosystems that have been found in these habitats (Hahn, 2006; Dole-Olivier et al., 2009; Robertson et al., 2009; Maurice et al., 2015). However, some geo-habitats did not follow this general pattern. For example, some fractured habitats have a very high DO concentration (Igneous Rock), while others have an unexpectedly high DOC concentration (Mudstones & Siltstones). Therefore, although these geologies are generally regarded as unsuitable habitats, the prevailing concentrations of at least some essential chemical parameters should be sufficient to sustain groundwater organisms. The few studies that have investigated fractured rocks, have found less diverse ecosystems than in other rock types (e.g. Hahn & Fuchs, 2009; Johns et al., 2015).

The 11 geo-habitats defined in this study differ significantly in hydrogeologicaL and hydrochemical characteristics, validating the lithology based typology. These key differences are likely to influence groundwater communities (Gibert et al., 1994; Datry et al., 2005; Hahn, 2006; Schmidt & Hahn, 2012), illustrating that it may not be appropriate to pool geologies into broader groupings. However, all of the key abiotic parameters are highly variable within geo-habitats, reflecting the general heterogeneity of groundwater habitats. This patchiness of resources is likely to be the determinant of the equally patchy distribution of groundwater fauna (Gibert et al., 1994; Datry et al., 2005). The high variability of abiotic parameters also illustrates that more detailed classifications are needed to explain faunal distributions in regional and local studies. For example, preferential flowpaths at the scale of 10^{-2}- 10^{2} metres may determine oxygen and food supply to microbes and metazoans in rocks (Stanford et al., 1994; Harvey, 1997; Larned, 2012). Therefore, incorporation of even more detailed hydrogeological data may be essential to explain ecosystem distribution at such scales.

Grouping of similar habitat types is important because it is impossible to sample 100's of habitats individually. Any habitat typology therefore needs to aim at a balance between minimising sampling units (habitats) and capturing the habitat differences that influence organisms. For a national scale study, the traditional habitat groups (karstic, porous, fractured) may be too coarse to accurately assess habitat suitability. Some fractured rock types in England harbour significant groundwater assemblages that would be overlooked using such typologies (e.g. see Hahn & Fuchs, 2009; Cornu et al., 2013). For example, fractured Igneous Rock in south-western England supports complex ecological communities, including *Niphargus* glenniei, the only endemic stygobite species in England and Wales (Knight, 2009; Johns et al., 2015).

For the national scale, the present typology with 11 geo-habitats appears to be a good compromise of feasibility and resolution, highlighting where the most abundant and diverse groundwater ecosystems are likely to occur in England and Wales. Similar hydrogeological grouping principles could be adopted to classify more highly resolved geo-habitats in other geographical areas.

2.5.2 Geo-habitat characteristics and quality

Of the two hydrogeological parameters investigated, transmissivity may be the better habitat quality proxy, with higher transmissivity indicating higher habitat quality. Transmissivity is a measure of permeability and integrates information on multiple scales. Because transmissivity is a measure of permeability, it informs on habitat chemistry (Hahn, 2006; Hahn & Fuchs, 2009; Robertson et al., 2009), the available habitat space, and may also give an indication of habitat connectedness and dispersal capacity. However, transmissivity data are obtained from borehole tests and therefore in highly karstic aquifers, where caves are likely to be the best habitats, transmissivity may be an inaccurate indicator of habitat quality. For example, although known to harbour abundant groundwater assemblages in extensive cave systems in England and Wales (Robertson et al., 2009; Johns et al., 2015), Highly Karstic Limestone had relatively low transmissivity (317.4 m^2/d) similar to that of Small-Pore Unconsolidated rock (252.1 m^2/d). However, it is a much better habitat than Small-Pore Unconsolidated rock, where most fauna is likely to be excluded due to small pore spaces (Jones et al., 2000).

Transmissivity is usually measured in successful abstraction boreholes, biasing available data to the more permeable sections of aquifers and omitting less permeable ones (Allen et al., 1997). Future groundwater studies may want to consider the number and distribution of abstraction points in a geology, as these may indicate the highly transmissive sections of the aquifers, and the number and frequency of suitable habitat patches.

Although porosity is important in determining habitat space and nutrient delivery in unconsolidated habitats (Hahn, 2006; Hahn & Fuchs, 2009), it is a poor proxy of habitat quality in all consolidated rocks. For example, the Chalk (34 %) is highly porous (Allen et al., 1997), but groundwater fauna are too large to live in the minuscule pore spaces (0.49 µm in the Chalk; Price et al., 1976).

Because of their isolation from the surface, groundwater ecosystems are thought to be oxygen-, nutrient- and solute-limited (Hahn, 2006; Tomlinson & Boulton, 2008; Schmidt & Hahn, 2012). DO, DOC, nitrate and calcium from the surface are therefore critically important to subsurface ecosystems, determining species distributions (Datry et al., 2005; Dole-Olivier et al., 2009), fuelling groundwater food webs (Mauclaire et al., 2000; Griebler & Lueders, 2009) and assisting in the maintenance of crustacean carapaces (Rukke, 2002). Although it is thought that more permeable rocks have higher levels of oxygen and nutrients, this was not always the case for the geo-habitats. While the hydrochemical data are probably representative for most geo-habitats, a sampling bias may have influenced some of the parameters in the limestones. For example, the mean DO in Mildly Karstic Limestone was relatively low, probably due to samples from confined areas, which are anoxic due to thick overlying strata (Allen et al., 1997). In contrast, the

highest mean DO concentration was recorded in Igneous Rock, a fractured habitat, which may indicate its suitability to groundwater ecosystems. The mean DOC in Highly Karstic Limestone was surprisingly low because, like transmissivity, hydrochemistry is measured in boreholes rather than caves, where DOC is likely to be higher (up to 4 mg/l, Simon et al., 2007; Ban et al., 2008). Mudstones & Siltstones had an unexpectedly high DOC concentration, indicating that sufficient nutrients for groundwater ecosystems are present in this geo-habitat. Furthermore, some non-karstic geo-habitats, such as Mudstones & Siltstones, also had surprisingly high mean calcium concentrations, most likely because of the frequent occurrence of calcareous strata (Jones et al., 2000). Overall, within some fractured geo-habitats (e.g. Igneous Rock, Mudstones & Siltstones) there appear to be areas with sufficiently developed fracture networks to produce suitable abiotic conditions for groundwater biota. While it is widely accepted that karstic geo-habitats such as the Chalk are good habitats supporting diverse assemblages (Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015), fractured geo-habitats are frequently described as being unsuitable habitats (Hahn & Fuchs, 2009) and acting as barriers to dispersal (Johns et al., 2015). The present hydrochemical data indicate this may not always be the case, and indeed four of the eight stygobite species have been recorded in the Mudstones & Siltstones (HCRS, 2016; Weitowitz et al., in prep.).

Overall, there are hydrochemical conditions in all geo-habitats in England and Wales that should be sufficient to support diverse groundwater communities. The mean concentration of hydrochemical parameters in all geo-habitats are above currently known thresholds for groundwater ecosystems. For example, the mean DO in all habitats is above 1 mg/l, the critical limit previously observed for stygobites (Hahn, 2006). The mean DOC concentrations are also above 0.4 mg/l, below which some faunal taxa may

disappear (Datry et al., 2005). Furthermore, the mean calcium concentrations in all geo-habitats are above 5 mg/l, the lower critical limit needed by surface water crustaceans (Rukke, 2002).

However, key quality differences between geo-habitats are likely to exist. Although all geo-habitats may occasionally harbour diverse communities, unsuitable habitat patches may be limiting in many places. Transmissivity, the best habitat quality surrogate, has minimum records of 1.8 m^2/d and lower in all geo-habitats. Such locations have very poorly developed fracture networks (Allen et al., 1997), most likely constraining the resources available to groundwater communities. Furthermore, all geo-habitats have areas where DO is below 1 mg/l and DOC is below 0.4 mg/l, indicating that invertebrates may not survive there (Malard & Hervant, 1999) or community diversity may be reduced (Datry et al., 2005). Geo-habitats with higher means of the parameters may provide suitable habitat conditions more frequently, while having fewer limiting patches. The high variability of abiotic parameters and the high proportion of sites below critical thresholds, provide evidence for the heterogeneity of groundwater habitats. Many studies maintain that the distribution of groundwater animals is shaped by the uneven distribution of resources, such as space, oxygen and nutrient availability (e.g. Deharveng et al., 2009), and the present data provide evidence for this small-scale heterogeneity. Taking the resource patchiness of aquifers into account therefore appears to be crucial in devising new habitat typologies, assessing habitat quality and understanding species distributions. However, this is difficult because measurements are biased towards the more permeable areas where water supplies can be obtained, and there is much less information on the location and extent of the less permeable areas.

As illustrated by the scoring system, geo-habitat quality varies and, for general discussion purposes, three broad quality categories (low, medium and high) may be distinguished. The geo-habitat scores are generally in agreement with the current knowledge of ecosystems in similar habitat types. Most fractured geo-habitats have lower quality scores, and they are often characterised by less diverse communities (Hahn & Matzke, 2005; Hahn & Fuchs, 2009; Johns et al., 2015). The Chalk has the best quality score and is known to harbour a significant proportion of groundwater biodiversity in the UK (Arietti & Edwards, 2006; Johns et al., 2015; Maurice et al., 2015).

However, the quality scores of other geo-habitats are not fully in agreement with what is known about their faunal communities in the literature. For example, Small-Pore Unconsolidated rock has a low quality, even though porous strata are generally considered to harbour diverse groundwater communities (e.g. Eberhard et al., 2005; Castellarini et al., 2007; Griebler et al., 2010). Although it has considerable permeability, its quality is limited by its small pore spaces, which may be as a low as 0.09 mm in fine sands (Cook, 2002). This will exclude all larger crustacean stygobites and limit ecosystem diversity and abundance. Pore size also influenced groundwater communities in previous studies (e.g. Dole-Olivier et al., 2009). Due to its high oxygen and nutrient concentrations Igneous Rock received an intermediate quality score in this study, supporting a previous study that found diverse assemblages in this geo-habitat, including significant numbers of the only English endemic stygobite, *N. glenniei* (Johns et al., 2015). In contrast, Igneous Rock in south-western England was classified as a non-aquiferous rock, and therefore as unsuitable habitat, in an earlier continental-scale study (Cornu et al., 2013).

Although habitat quality increases from fractured, to porous to karstic geo-habitats in the scoring system, several exceptions (Igneous Rock, Small-Pore Unconsolidated) illustrate that such broad groupings may be inappropriate to assess species distributions at the national scale. Instead, typologies accounting for more geological variability may be better suited to capturing the subterranean heterogeneity. The geo-habitat scores account for the relative proportion of good and bad habitat patches in terms of their hydrogeological and hydrochemical properties. However, the hydrochemical requirements and tolerances of groundwater organisms, and in particular critical thresholds, are not well known (Larned, 2012). While these scores are a start to exploring groundwater habitat quality quantitatively, further research is needed to develop new methods of incorporating geological heterogeneity in habitat assessments and to explore what habitat quality entails for groundwater organisms.

2.5.3 Distribution of geo-habitats

The geo-habitats defined in this study differ greatly in distribution and their level of connectedness. Over 60 % of England and Wales are covered by poor fractured groundwater habitats, such as Metamorphic Rock and Mudstones & Siltstones. This increases the relative ecological significance of high-quality habitats, such as the Chalk and Moderately Karstic Limestone that harbour a significant proportion of groundwater assemblages in England (Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015).

Groundwater geo-habitats differ in their spatial distribution, ranging from discrete to continuous. Some karstic geo-habitats occur in north-south trending connected belts, whereas fractured geo-habitats, such as Igneous and Metamorphic Rock, are much more

disjointed. The low hydrological connectivity is critical in habitats, where dispersal constraints are one of the most important distribution determinants, and are responsible for the distribution ranges of groundwater fauna remaining static for long periods of time (Culver et al., 2009; Galassi et al., 2009; Robertson et al., 2009; McInerney et al., 2014; Johns et al., 2015).

In south-western England and Wales, suitable habitats (e.g. Igneous Rock, Highly Karstic Limestone) occur in relatively small patches or as isolated cave systems. These habitats are known to harbour complex ecosystems and abundant stygobites (Robertson et al., 2009; Johns et al., 2015), but dispersal opportunities are likely to be limited compared to the extensive karstic belts in northern, central and eastern England. High transmissivities in the karstic belts occur frequently, suggesting better overall habitat connectivity and facilitated species mobility compared to the disjointed habitats in south-western England. Therefore, at the national scale high-connectivity karstic geo-habitats may be an important influence on the observed species distributions, providing important migratory routes for groundwater fauna. However, despite this many species are absent from northern England (Robertson et al., 2009; Maurice et al., 2015), which may be due to extirpations in the Devensian glaciation combined with the low dispersal rates of stygobites (Robertson et al., 2009).

In contrast to the karstic belts, many other potentially good habitats such as Large-Pore Unconsolidated rock and Mixed Sandstone, are limited to relatively small, isolated patches throughout England, often separated by bands of poor quality habitats, such as mudstones. Diverse groundwater communities may not develop in many such isolated places, because species cannot disperse there.

Overall, two main forces may shape species distributions at the national scale: High-quality karstic habitats may provide preferential dispersal corridors on the north-south axis, while the intermittent extensive outcrops of low-quality fractured habitats may limit species movement on the east-west axis. An example for this may be the endemic *N. glenniei*, which is limited to patches of suitable rock in south-western England. Low-quality habitats may obstruct its migration to central England (Johns et al., 2015).

Hyporheic zones and some of the more permeable superficial deposits (e.g. alluvium in northern England; Smedley et al., 2004) are likely to provide important additional habitats for groundwater organisms. For example, *N. aquilex* may use these 'highways' for faster dispersal ('hyporheic corridor concept', Ward & Palmer, 1994; Johns et al., 2015). However, these habitats were not considered in the analysis, because chemistry data for them was not available and little hydrogeological information is available for superficial deposits.

Stygobites are characterised by a restricted ability to disperse and this feature clearly limits their ability to use habitats that have sufficient quality. Finding more robust quantitative measures of (trans-aquifer) connectivity, alternatives to transmissivity and porosity, is an important next step in explaining species distributions across multiple spatial scales (e.g. Larned, 2012). While water chemistry is clearly important to groundwater ecosystems, low habitat connectivity may override this because biota cannot reach potentially suitable habitat patches.

2.6 Conclusion

This paper developed and tested a new national-scale habitat typology for England and Wales, in which 11 geo-habitats were defined. These were significantly different in hydrogeological and hydrochemical characteristics, validating the categorisation process and indicating this may be a suitable number of categories for national-scale evaluations.

However, despite the higher geological resolution used, there was considerable within-habitat variation in all parameters. This illustrates the patchiness and heterogeneity of groundwater habitats, and the need to use higher resolution geological data to explain species distributions in both regional and local groundwater ecosystem studies.

A method was developed to determine the overall quality of geo-habitats using critical thresholds required by groundwater ecosystems, and taking into account the relative proportion of suitable and unsuitable habitat patches. This could be an appropriate method for assessing habitats in other areas, using existing hydrogeological and hydrochemical data. It is also a method that is applicable at local, regional and national scales.

Substantial parts of England and Wales are covered by low-quality, mainly fractured, geo-habitats that provide limited space and little connectivity for dispersal, and probably limit dispersal on an east-west axis. Medium- and high-quality geo-habitats, mainly karstic and porous in nature, occur predominantly in eastern and southern England. These provide extensive space, suitable water chemistry and connected corridors on a north-south axis. Most biodiversity is likely to be restricted to these karstic

hotspots, which potentially should be prioritised in conservation, particularly in the light of the low UK species diversity.

Overall, the presented habitat typology provides a suitable tool for assessing groundwater ecosystem distribution at a national scale. The definition and quality assessment of the geo-habitats provide a framework for the next step: Assessing how geology is affecting groundwater ecosystem distributions in England and Wales. Typologies with higher geological detail and explanatory power may also be used to study such ecosystems in other areas, giving this study the scope of a wider applicability.

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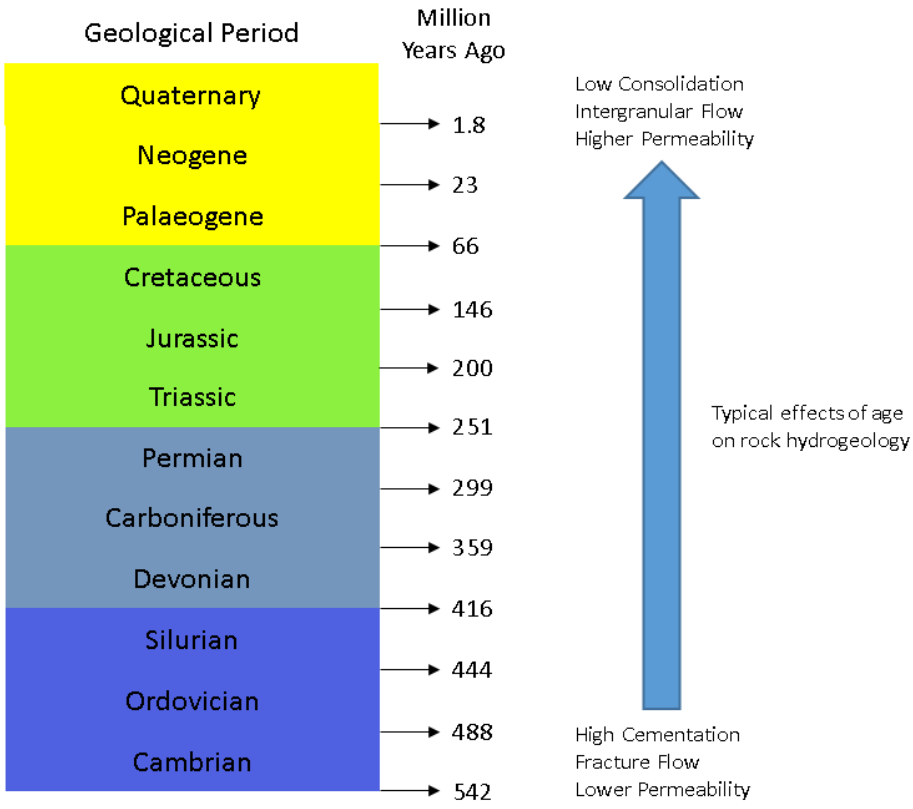
2.9 Appendices

Appendix 2.1: Summary of previous studies that have grouped groundwater habitats. Stated are the type of grouping method, the number of habitats defined, the scale of analysis and whether significant differences in faunal assemblages were found between them.

Authors	Grouping method based on	Number of Habitats	Scale	Significant difference in stygobite assemblages
Castellarini et al. (2007)	Hydrogeology – mainly water flow type	4 (karstic, coarse porous, morainic alluvial, fine porous)	Regional – Jura mountains (France) (1,273 km^2)	Yes
Dole-Olivier et al. (2009)	Hydrogeology – mainly surface hydrological exchange	2 (karstic & porous)	Regional – Jura mountains (France) (1,273 km^2)	Yes
Hahn & Fuchs (2009)	Hydrogeology – mainly lithology	4 (karstic, porous, fractured, compact)	Regional – County Baden-Wuertemberg (Germany) (37,750 km^2)	Yes
Robertson et al. (2009)	Hydrogeology – mainly lithology and hydrological exchange	7 (Chalk, Carboniferous Limestone, Jurassic Limestone, Fractured Sandstone, Lower Greensand, alluvium, gravel)	Country – United Kingdom (243,610 km^2)	Yes
Cornu et al. (2013)	Hydrogeology – mainly pore sizes and permeability	12 (low-high permeability consolidated habitats, low-high permeability unconsolidated habitats)	Continent – Europe (10,180,000 km^2)	Yes
Johns et al. (2015)	Hydrogeology – mainly lithology and water flow type	5 (granular, igneous/metamorphic, mudstone/siltstone, sandstone, carbonate)	Regional – Devon & Dorset (UK)	Yes
Present Study	Hydrogeology – mainly lithology, permeability, degree of consolidation, nature of water flow Hydrochemistry – differences in key parameters	11 (see Fig. 1)	Country – England & Wales (152,740 km^2)	Not yet tested



Appendix 2.2: Diagram showing geological ages involved in creating UK bedrock. Younger strata tend to be less consolidated and promote increased permeability (shown by the arrow).

Appendix 2.3: Showing the broad aquifer types, area cover in km^2 and total area cover in % of the geo-habitats.

Geo-Habitat	Broad Aquifer Type	Area Cover (km^2)	Area Cover (%)
Chalk	Karstic	19,201	12.6
Highly Karstic Limestone	Karstic	3,151	2.06
Moderately Karstic Limestone	Karstic	4,497	2.94
Mildly Karstic Limestone	Karstic	1,472	0.96
All Karstic	**Karstic**	**28,321**	**18.54**
Small-Pore Unconsolidated	Porous	13,733	8.99
Large-Pore Unconsolidated	Porous	1,490	0.98
Mixed Sandstone	Mixed	12,747	8.35
All Porous & Mixed	**Porous / Mixed**	**27,970**	**18.31**
Fractured Sandstone	Fractured	27,817	18.21
Igneous Rock	Fractured	3,621	2.37
Metamorphic Rock	Fractured	3,777	2.47
Mudstones & Siltstones	Fractured	61,234	40.09
All Fractured	**Fractured**	**96,449**	**63.15**
All	**All**	**152,740**	**100**

Appendix 2.4: Summary statistics for the principal component analysis showing correlations of quantitative variables and r^2 values of qualitative variables for the two principal component axes with Eigenvalues > 2.

Component	Quantitative Variable	Correlation / r^2	P	% Variance Explained
Principal Component 1	Calcium	0.6	< 0.001	16 %
	Nitrate	0.51	< 0.001	
	DOC	0.48	< 0.001	
	DO	0.1	< 0.001	
	Geology	0.86	< 0.001	
	Transmissivity	0.83	< 0.001	
Principal Component 2	DO	0.54	< 0.001	13.8 %
	Nitrate	0.17	< 0.001	
	DOC	0.09	< 0.001	
	Calcium	-0.21	< 0.001	
	Geology	0.93	< 0.001	
	Transmissivity	0.91	< 0.001	

Appendix 2.5: Summary of UK geo-habitat quality scores sorted from highest to lowest.

Geo-Habitat	log Habitat Quality Score
Mildly Karstic Chalk	4.21
Moderately Karstic Limestone	3.66
Mildly Karstic Limestone	3.1
Highly Karstic Limestone	3.07
Mixed Sandstone	2.98
Large-Pore Unconsolidated	2.69
Igneous Rock	2.66
Mudstones & Siltstones	2.46
Small-Pore Unconsolidated	2.36
Fractured Sandstone	2.16
Metamorphic Rock	1.68

Chapter 3 – The groundwater ecology of four poorly sampled geologies in England and Wales

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3.1 Abstract

Obligate groundwater taxa (stygobites) provide a significant contribution to global species diversity and are components of ecosystems that deliver essential services, such as the provision and storage of clean drinking water. However, the knowledge of UK stygobite species distributions and the environmental factors governing faunal communities remains incomplete. The primary aim of this paper was to increase the UK sampling coverage, providing the first samples in two sandstones, Devonian Sandstone and Lower Greensand, while extending sampling coverage of two limestones, Jurassic and Magnesian Limestone. The objective was to provide the first overview of the invertebrate ecology of these important UK aquifers and to assess the processes that shape their faunal communities. The environmental controls on copepod biomass and abundance have also rarely been studied in the UK. A secondary aim was therefore to assess some of the abiotic drivers of copepod biomass and abundance, including geology, site type, physical site cover, presence of superficial deposits, and distances to surface water and past glacial limits. A total of 34 sites in the four geologies were sampled on a single occasion using phreatic sampling nets, and stygobites were identified to species level while other freshwater and terrestrial taxa were identified to broad taxonomic group. The biomass of all fauna were calculated using geometric formulae or length-weight regressions from the literature.

Overall, the investigated geologies clearly differ in their significance as groundwater habitats. Sampling detected *M. leruthi* at one site in the Magnesian Limestone (southern outcrop), while *N. aquilex* occurred at several sites in the Jurassic Limestone (northern outcrop). In the Devonian Sandstone two species occurred at few

sites, while no stygobites were documented in the Lower Greensand. The drivers of faunal distributions appear to differ between limestones and sandstones. While the limestones provide good habitat throughout their entire outcrops, they are located in northern England, making past glaciations and dispersal the most likely stygobite distribution controls. In contrast, the sandstones are located in southern England and Wales adjacent to geologies that harbour high stygobite diversity, indicating that stygobites are most likely controlled by geological features (e.g. fracture density and pore spaces).

The biomass in geologies was dominated by terrestrial carbon, indicating that the cross-ecosystem flux of carbon is probably important in sustaining groundwater communities in boreholes and wells. Stygobites contributed most biomass in the limestones, in which only sealed sites were sampled, reducing the likelihood of terrestrial carbon falling in. Therefore, the higher proportion of stygobites is likely to be an aquifer-specific effect, indicating a stygobite preference for limestone habitats. Site-specific characteristics were found to be the main predictor of copepod biomass and abundance, whereas aquifer geology exerted little influence. This highlights the need for a better understanding of site characteristics and the use of high geological resolution in groundwater ecosystem studies.

3.2 Introduction

Given its abiotic characteristics and relative isolation from the surface, the subterranean realm is one of the most unique habitats on earth. Groundwater life has needed to adapt to particular conditions, such as the absence of light, low nutrient conditions and stable temperatures (Gibert et al., 1994; Schmidt & Hahn, 2012). For example, obligate subterranean invertebrates (stygobites) have developed unique adaptations, such as low metabolic rates, complex sensory systems, and longer life cycles to survive in this challenging environment (Coineau, 2000; Humphreys, 2006). Stygobitic fauna, which is largely dominated by crustaceans, also contributes a significant proportion to the global species pool (Deharveng et al., 2009) with high levels of endemism observed in most regions (Culver & Sket, 2000; Christman et al., 2005). Many distribution studies, such the PASCALIS project, have been carried out in mainland Europe and the USA (Humphreys, 2006; Deharveng et al., 2009; Dole-Olivier et al., 2009a; Gibert et al., 2009; Stoch et al., 2009; Culver & Pipan, 2011), but only few have been undertaken in England (Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015). These have increased our understanding of species distributions in groundwater, but gaps in the knowledge of subterranean assemblages still exist (Robertson et al., 2009) and the ecology of many geological habitats remains poorly understood.

The geological formations and the associated groundwater are the principal habitat for stygobites. Most studies on groundwater ecosystems concluded that geology is a major control on the distribution of stygobites (Hahn, 2006; Dole-Olivier et al., 2009a; Hahn & Fuchs, 2009, Griebler et al., 2010). Primarily, geo-morphological features (e.g. pore spaces, fractures, caves) determine the amount of space available to stygobites

(Botosaneanu, 1986; Datry et al., 2005; Dole-Olivier et al., 2009a; Maurice & Bloomfield, 2012), and thus may limit the abundance and size structure of communities. Geology also determines hydrological connectivity, which in turn affects the concentrations of nutrients and oxygen in the subsurface (Hahn, 2006; Maurice & Bloomfield, 2012; Schmidt & Hahn, 2012). Aquifers are highly heterogenous (Griebler & Mösslacher, 2003; Larned, 2012), resulting in the uneven distribution of stygobites in their habitats (Mösslacher, 2003). This heterogeneity further results in low dispersal capacities of stygobites (Johns et al., 2015), and is ultimately responsible for the high levels of endemism in groundwater habitats (Culver & Sket, 2000; Culver et al., 2009). Overall, geology is of central importance to stygobitic distributions because it determines the physical and chemical habitat quality, as well as the ability of species to disperse to new geographic regions.

Current distribution studies are clearly biased towards the more easily accessible limestone habitats, such as caves and karst springs (Gibert & Culver, 2009). In the UK primarily the Chalk has been sampled, partly because of its importance for the public water supply (Price, 1985; Arietti & Edwards, 2006). It is now well established that the Chalk and Carboniferous Limestone are good habitats for stygobites, both in terms of abundance and species diversity (Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015). Other geological habitats, however, are scarcely sampled (Jurassic Limestone, Mudstones & Siltstones) or lack sampling altogether (Magnesian Limestone, Lower Greensand). Papers have called for investigations on sandstones (Larned, 2012), which have rarely been sampled (e.g. Hahn & Fuchs, 2009; Johns et al., 2015). This paucity of data on some geological formations limits coherent distribution assessments at the national UK scale. Due to the heterogeneity of groundwater habitats and the

unpredictability of species distributions, thorough sampling coverage is essential to reliably determine species presence (Dole-Olivier et al., 2009b; Larned, 2012).

The main aim of this paper was to improve the sampling coverage of UK geologies, and to provide a first assessment of the invertebrate ecology of four important UK aquifers and their significance as habitats. Overall, two karstic habitats, the Jurassic and Magnesian Limestone, and two sandstone habitats, the Lower Greensand and the Devonian Old Red Sandstone (referred to in the following as Devonian Sandstone), were sampled. The study integrates new sampling data with previously collected unpublished samples from the British Geological Survey (BGS) and the Hypogean Crustacea Recording Scheme (HCRS). According to their scores in a recent habitat typology (chapter two, Weitowitz et al., submitted, two higher quality karstic habitats were compared with two relatively poor sandstone habitats. Both karst aquifers are characterised by occasional caves, but frequent solutional enlargement of fractures to form networks of small conduits and fissures (Atkinson & Smart, 1981; Worthington & Ford, 2009). The resulting high transmissivities (Allen et al., 1997) should provide suitable habitat conditions for stygobites. In contrast, Devonian Sandstone is a rock in which permeability is limited to a network of smaller fractures, while in the Lower Greensand most of the transmissivity occurs in unconsolidated sands (Allen et al., 1997). The habitat quality of the sandstones should be sufficient to harbour some stygobites, but in lower diversity and abundance than the limestones (see Hahn & Fuchs, 2009; Weitowitz et al., submitted).

Groundwater ecosystem studies usually investigate the presence and absence or abundance of species in subterranean habitats (Dole-Olivier et al., 2009a; Hahn & Fuchs, 2009; Stoch et al., 2009). Surprisingly little attention has been given to biomass, which is

the energy currency driving ecosystems (Reiss & Schmid-Araya, 2008, 2010). The distribution of biomass across trophic levels is essential for many ecosystem processes, such as predator-prey cycles, and determines the amount of energy available to other levels in the food chain (Momot, 1995; Bowen, 1997). Stygobites are top-level predators in groundwater food webs, feeding on diverse trophic elements such as microbes, protozoans and copepods (chapter five; Boulton et al., 2008). The availability of biomass in groundwater habitats benefits macrofauna, which consume biomass and extract energy from it. Using biomass as a response variable may provide researchers with an additional tool to assess environmental controls in groundwater ecosystems. Because biomass is a reflection of the resource availability in a habitat, it may also act as a proxy of groundwater habitat quality. The secondary aim of the paper was therefore to quantify the total biomass in the four geologies. Furthermore, the relative biomass contribution of stygobites, other freshwater taxa and terrestrial taxa to the total carbon pool was quantified.

While groundwater animals in boreholes are influenced by geology, they are also likely to be affected by a range of other environmental variables (e.g. distance to glacial limit and surface water, Mösslacher, 1998; Galassi et al., 2009; Robertson et al., 2009; superficial deposits; Maurice et al., 2015) and site-specific characteristics, such as depth (Strayer, 1994; Hakenkamp & Palmer, 2000; Dumas et al., 2001), diameter (Maurice & Bloomfield, 2012) and the type of physical constructed cover. Although the list of potentially influential determinants is long, most are probably related to resource provision in an energy limited environment (Gibert et al., 1994; Hahn, 2006). For example, deep sites may restrict groundwater communities because they are recharged with groundwater depleted of nutrients and oxygen (Datry et al., 2005; Schmidt & Hahn,

2012). However, in fractured habitats deeper sites may also intercept more habitat features, potentially receiving more organisms (Maurice & Bloomfield, 2012). Distance to surface water may be important because it regulates the hydrological connectivity with surface water bodies, which likely determines nutrient and oxygen concentrations in the subsurface (Hahn, 2006; Bork et al., 2009). It has also been documented that stygobites are absent from areas confined by overlying superficial deposits (Maurice et al., 2015), most likely due to restricted water circulation and near-anoxic conditions (Smedley et al., 2003, 2004). Only few of these parameters have been assessed in the UK and their effect on the biomass of groundwater animals has not been studied. Thus the third aim was to assess the relative influence of geology, environmental factors (distance to surface water and past glacial limits) and site-specific characteristics (depth, constructed physical cover of site) on individual copepod biomass and abundance.

3.3 Methodology

3.3.1 Distribution of fauna

Selection of geologies and sampling areas

Initially all UK sampling records from the Hypogean Crustacea Recording Scheme (HCRS) were consulted to evaluate where sampling coverage was poor and which geologies warranted further sampling. When choosing geologies to sample, the number of existing samples relative to habitat size was initially assessed. The Chalk and the Carboniferous Limestone have been sampled extensively in past groundwater studies and are known to be important habitats for stygobites in the UK (Arietti & Edwards, 2006; Robertson et al.,

2009; Knight, 2011; Maurice et al., 2015). Igneous and Metamorphic Rock were not sampled further because of their small outcrop areas. The proposed habitat quality was also considered in the selection process. Although they cover a vast area, Mudstones & Siltstones were not sampled because they are considered poor habitats for macrofauna (chapter two; Johns et al., 2015).

Two karstic geologies with relatively prominent outcrop areas were identified as having poor sampling coverage. Magnesian Limestone occurs as a relatively short belt in northern England (Fig. 3.1). All five previous BGS samples were collected in the outcrop north of the Devensian glacial limit and did not detect stygobites. The aim was to sample in its southern outcrop to establish if groundwater fauna is present in the Magnesian Limestone. Jurassic Limestone extends in a belt from northern to southern England (Fig. 3.1). Previous sampling in the southern outcrop was undertaken by the BGS, and results are available in the HCRS, along with 2 sites from further north (Fig. 3.1). Overall, species diversity and abundance in the southern outcrop were high. The aim in this study was to obtain samples from the northern outcrop, which would be expected to harbour similar species diversity because the aquifer provides a continuous dispersal corridor on the north-south axis.

The two sandstone geologies were selected because they have not been investigated previously. Lower Greensand is a type of mixed flow sandstone that occurs as a continuous circular belt in south-eastern England adjacent to the Chalk (Fig. 3.1). Devonian Sandstone is a fractured sandstone that is a spatially heterogenous aquifer on the boarder of England and Wales (Fig. 3.1). Sampling in the UK and other countries has been conducted in similar geologies: For example, several stygobite species were found in

Devonian and Permo-Triassic Sandstone in Devon and Dorset (Johns et al., 2015), and are also reported from fractured sandstones in Germany (Hahn & Fuchs, 2009). The aim was to sample both of these sandstones to establish whether they are suitable habitats for stygobites.

Field sampling

Samples were obtained on a single occasion from each of the 34 sites, which are part of a UK groundwater monitoring network maintained and regulated by the UK Environment Agency. 18 sites were sampled in the Devonian Sandstone, 7 sites in the Lower Greensand, 5 sites in the Jurassic Limestone (northern outcrop) and 4 sites in the Magnesian Limestone (southern outcrop). For the latter three geologies only a limited number of sites were available for sampling. Due to reliance on existing groundwater access points it was not possible to implement a statistically randomised design or to be selective about possibly influential borehole parameters such as site type, width or depth (see Table 3.1 for sampling site characteristics). Sampling sites were considered to be 'closed' when a completely sealed cover was present, and considered 'open' when a cover was partial or completely absent.

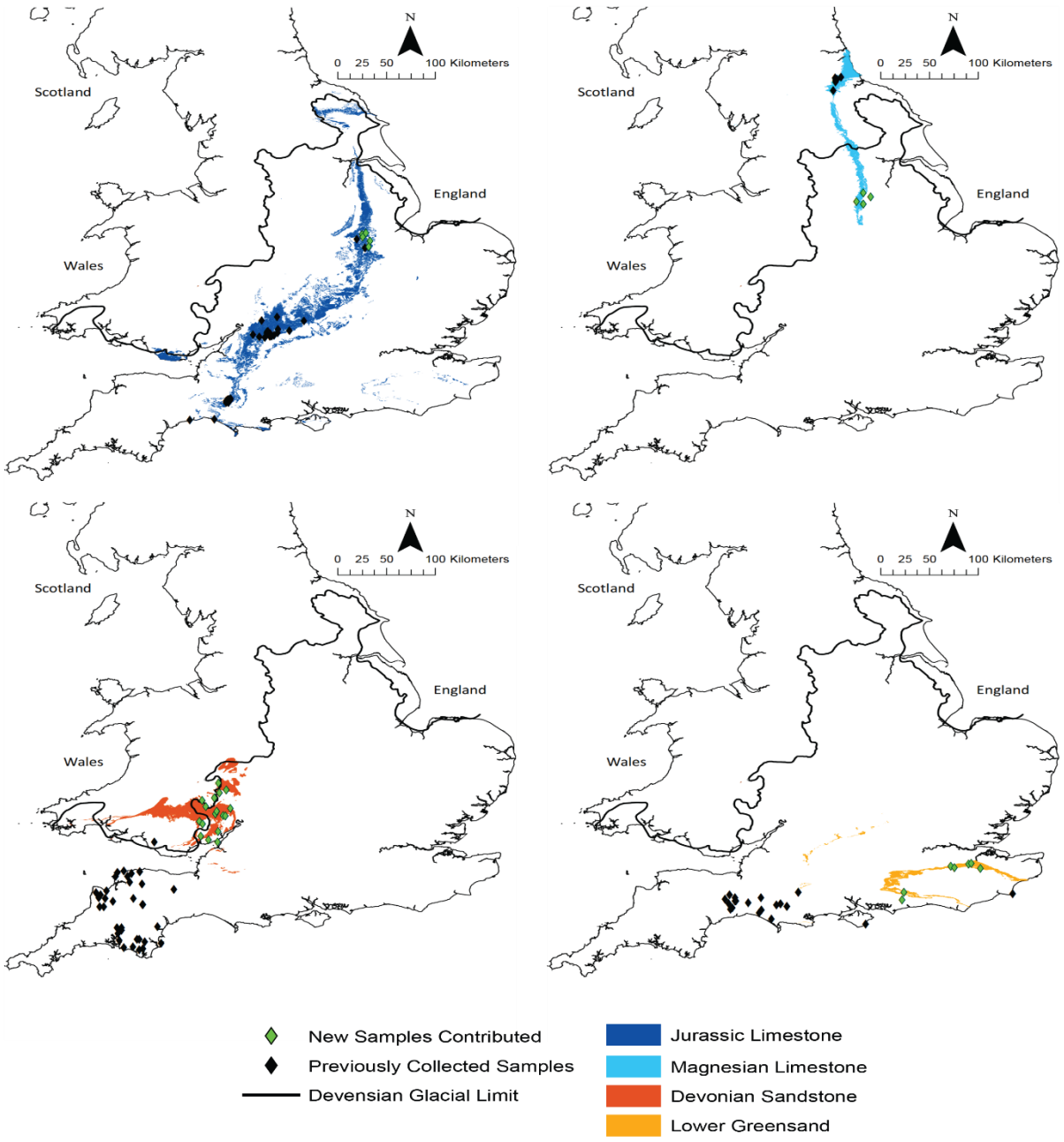

Fig. 3.1: Distribution of the sites sampled for this study and samples collected previously in the four geologies. No prior samples were collected in Devonian Old Red Sandstone and Lower Greensand, but similar lithologies have been sampled in England.

Invertebrates were sampled using a weighted plankton net (Cvetkov, 1968). A mesh size of 63 μm was used to capture all meio- and macrofauna. The net diameters were selected according to site diameter and varied between 3 and 30 cm. On each sampling occasion the plankton nets were connected to a cord on a reel. As in previous groundwater invertebrate studies (Johns et al., 2015; Maurice et al., 2015) the net was then lowered to the bottom of the site, slightly raised and lowered three times to disturb the sediment, and to agitate fauna potentially resting at the bottom (see Hancock & Boulton, 2009). These samples integrate fauna from the bottom and the water column as the net moves upwards through the water. Three net samples were collected from each site to ensure that a representative proportion of the aquatic fauna was captured. Once on the surface the contents of each net haul were immediately washed into vials with > 95 % ethanol for preservation until the start of laboratory sorting.

Laboratory sorting and identification

Because of the large amount of sediment in samples, fauna were separated from the sediment using the flotation technique (Anderson, 1959). Samples were immersed in a sugar solution with a specific gravity of 1.12, and fauna floated to the surface of the solution due to their lower specific gravity. The sugar solution was then decanted and the remaining material immediately sorted under a light microscope. A higher resolution compound microscope was used to verify the identity of small species (e.g. *M. leruthi*) or juveniles of *N. fontanus*. Stygobitic amphipods were identified to species level using freshwater invertebrate identification keys (Gledhill et al., 1993; Knight & Gledhill, 2010). Copepods were not identified further and it remains unknown whether they are

stygobitic, stygophilic or stygoxenic. Most other aquatic fauna were only identified to coarse taxonomic level, such as Oligochaeta or Arenae.

Assessing species distributions

ArcGIS was used to map stygobite species and copepod distributions on the outcrop of the sampled geologies. Faunal distributions were set in the context of available information on the lithology and transmissivity of the geologies, to determine the factors most likely controlling the observed patterns. Very little is known about the dispersal rates of groundwater organisms, but dispersal is thought to be extremely slow due to the heterogeneity of rock. A species was defined as being 'within range' of a sampling site if it occurred within 20 km or in other geologies to the north, east and west of this site. This definition acknowledges that stygobite distributions in northern Europe are influenced by past glaciations, and a species is only expected at a site if it has been observed as least as far north as the site itself.

Table 3.1: Summary of the number, type and cover (open or sealed) of sampling sites in the four geologies. Also shown are the mean and range of other borehole parameters, such as depth, water level, diameter and transmissivity (Allen et al., 1997; Jones et al., 2000). bgl = below ground level, T = transmissivity

Geology	No Bhs	No Wells	No Closed	No Open	Mean Depth (m)	Min Depth (m)	Max Depth (m)	Mean Water Level (m bgl)	Min Water Level (m bgl)	Max Water Level (m bgl)	Mean Water Column (m)	Min Water Column (m)	Max Water Column (m)	Mean T (m^2/d)	Min T (m^2/d)	Max T (m^2/d)
Jurassic Limestone	5	0	5	0	40.6	21	53.1	16.5	5.5	25.5	24.2	5.4	43.5	665	1	14,000
Magnesian Limestone	4	0	4	0	42.3	10.9	80.1	19.8	4.2	55.3	22.6	4.2	54.6	233	6	4,300
Devonian Sandstone	14	4	14	4	38.8	1	74.6	13.4	0.3	48.6	25.4	0.5	55.3	51	< 0.01	350
Lower Greensand	1	6	3	4	36.5	20.5	58	24.5	2.8	38.1	12.1	0.04	55.1	270	33	3,400

3.3.2 Calculating individual and total biomass

Individual biomass

The length and width of every individual was measured using an eyepiece graticule under a light microscope. The body size measurements were then converted to individual biovolume or freshweight using previously published linear regression equations and geometric formulae (see Table 3.2). Following Reiss & Schmid-Araya (2008) faunal biovolume was converted to freshweight assuming a specific gravity of 1.1. Individual carbon content in µg carbon (µg C) was then estimated assuming dry-wet weight ratio of 0.25 and a dry weight carbon content of 40 % (Feller & Warwick, 1988; Reiss & Schmid-Araya, 2008).

Total biomass in geologies

To calculate the total biomass in the sampled geologies the biomass of all individuals (stygobites, freshwater fauna and terrestrial fauna) was summed. Because the sampling effort differed between geologies, the cumulative biomass was standardised by dividing by the number of sampling sites in each geology. Furthermore, the relative proportion of stygobites, copepods, freshwater fauna and terrestrial fauna was calculated. Except for copepods, which were considered separately, freshwater fauna included all aquatic freshwater fauna that also occur in surface water ecosystems, including water mites, isopods, daphnids, nematodes, oligochaetes and trichoptera larvae. Terrestrial fauna included all taxa that do not complete their life cycle in water and must have either been carried in by water or accidentally fallen into the sites. These included the taxa Arenae, Collembola, Coleoptera, Diptera, Hymenoptera and Orthoptera (see Table 3.2 for list of taxa). Due to time constraints, microbes and protozoans, important components of

groundwater ecosystems (Sinclair & Ghiorse, 1987; Griebler & Lueders, 2009; Griebler et al., 2010), were not considered in the biomass analysis.

Table 3.2: Taxon-specific linear regressions and geometric formulae used to convert body dimensions (in mm) of taxa found in boreholes and wells into biovolume (in nL or mL) or freshweight (in mg). a = body length (mm), b = body with (mm)

Taxon	Biovolume (V) or Freshweight (Fwt)	Reference
Acari	$V\ (nL) = a * b^2 * 399$	Reiss & Schmid-Araya (2008)
Amphipoda	$Fwt\ (mg) = 0.0058 * a^{3.015}$	Benke et al. (1999)
Arenae	$Fwt\ (mg) = 0.1261 * (ab)^{1.408}$	Gruner (2003)
Cladocera	$V\ (mL) = (ab^2 * \pi) / 6$	Reiss & Schmid-Araya (2008)
Collembola	$Fwt\ (mg) = 0.0601 * (ab)^{1.374}$	Gruner (2003)
Copepoda	$V\ (nL) = a * b^2 * 560$	Feller & Warwick (1988)
Coleoptera	$Fwt\ (mg) = 0.0077 * a^{2.910}$	Benke et al. (1999)
Diptera	$V\ (mL) = (ab^2 * \pi) / 6$	Reiss & Schmid-Araya (2008)
Entognatha	$Fwt\ (mg) = 0.0923 * (ab)^{1.346}$	Gruner (2003)
Isopoda	$Fwt\ (mg) = 0.0578 * (ab)^{1.351}$	Gruner (2003)
Hymenoptera	$0.56 * a^{1.56}$	Sabo et al. (2002)
Nematoda	$Fwt\ (mg) = a * b^2 / 1\ 600\ 000$	Andrassy (1956)
Oligochaeta	$V\ (nL) = a * b^2 * 530$	Feller & Warwick (1988)
Orthoptera	$0.03 * a^{2.55}$	Sabo et al. (2002)
Trichoptera	$Fwt\ (mg) = 0.00408 * a^{2.82}$	Johnston & Cunjak (1999)

3.3.3 Assessing environmental predictors of copepod biomass and abundance

All data analysis was conducted in R, a community-based software for statistical computing (R Development Core Team, 2016).

Overall, the effects of geology, site type, physical site cover, superficial deposits, season, distance to surface water and past glacial limit, site depth and depth to water table on both mean copepod biomass (826 measured individuals) and abundance were tested. Initially, the effect of site type on copepod responses was considered, because site types (boreholes / wells) were not sampled evenly in all geologies (e.g. in limestones only boreholes sampled). Kruskal-Wallis tests indicated that site type significantly influences both copepod biomass and abundance.

Sampling site had a strong influence on copepod biomass, indicating that uncontrolled site-specific characteristics influence copepods in the sampled geologies. Since copepods represent non-independent samples from the same site being subject to the same uncontrolled site-specific factors, sampling site was considered as an influential random factor. For the response copepod biomass, linear mixed-effects models with a logarithmic link function were run for each predictor in the R package 'lme4' (Bates et al., 2015). Prior to the analysis, the biomass variable was log10 transformed, to solve the heterogeneity in the residuals of the models. Site type (boreholes / wells) did not have an effect on copepod biomass in the mixed-effects model and therefore all copepod biomass data were analysed collectively. Model assumptions were checked by assessing the independence of sampling sites, the normality and homogeneity of residuals, and overdispersion (see Appendix 3.1). The R package 'piecewiseSEM' (Lefcheck, 2015) was used to extract the explained variance (r^2) of fixed and random effects from the models

(also see Nakagawa & Schielzeth, 2013; Johnson, 2014). The biomass analysis was also repeated separately for borehole and well data, showing the same trends and therefore only the complete analysis is shown.

For the response copepod abundance, linear and generalised mixed-effects models did not fit the data. Therefore, non-parametric Kruskal-Wallis tests and Kendall's tau correlations were used to assess the influence of environmental predictors. Due to the confounding effect of site type (boreholes / wells) mentioned above, borehole and well data were split for separate analysis.

3.4 Results

3.4.1 Faunal distributions in geologies

Three stygobite species (*N. aquilex*, *N. fontanus* and *M. leruthi*) and copepods were found in the 4 sampled geologies (Figs. 3.2, 3.3).

Previous sampling in the Jurassic Limestone indicated that it harbours *N. aquilex* (Fig. 3.2a), *N. fontanus* (Fig. 3.2b), *M. leruthi* (Fig. 3.2c), copepods (Fig. 3.3a), *N. kochianus* (Fig. 3.3b) and *P. cavaticus* (Fig. 3.3c) in its southern outcrop. Additional sampling in the northern outcrop documented relatively high abundances of *N. aquilex* (up to 11 individuals) (Fig. 3.2a), but did not detect the species present in the southern outcrop and other karstic geologies to the north, east and west, including *N. kochianus*, *N. fontanus*, *M. leruthi* and *C. subterraneus* (Table 3.3). Copepods occurred at high abundances throughout the entire Jurassic Limestone belt (Fig. 3.3a). Both freshwater and terrestrial

taxa (incl. Coleoptera, Diptera, Orthoptera and Collembola) were frequently present at relatively high abundances (Tables 3.4, 3.5).

In the Magnesian Limestone *M. leruthi* were found in the southern outcrop (Fig. 3.2c), whereas both *C. subterraneus* and *N. aquilex* that occur in other geologies in northern England, were absent (Table 3.3). Previous sampling did not detect stygobites in the outcrop north of the Devensian glacial limit. Copepods occurred at high abundances throughout the entire Magnesian Limestone outcrop (Fig. 3a). In comparison to the other geologies few other freshwater or terrestrial taxa were found, and they occurred at low abundances (Tables 3.4, 3.5).

With two stygobite species the Devonian Sandstone contained the highest species diversity in this study. *M. leruthi* occurred at 22 % of sites with abundances of up to 60 individuals (Table 3.4, Fig. 3.2c). Three juvenile *N. fontanus* were sampled from one of the boreholes (Fig. 3.2b). Although occurring in other geologies less than 20 km away, *N. aquilex* and *P. cavaticus* were not detected (Table 3.3). Copepods were widespread and abundant in this sandstone with up to 116 individuals at one site (Fig. 3.3a). Other freshwater and terrestrial taxa in Devonian Sandstone were abundant, but their occurrence was generally limited to few sites (Tables 3.4, 3.5).

Although the Lower Greensand lies within the range of six stygobite species that occur in the nearby Chalk (Table 3.3), which is directly adjacent and in hydrological continuity with the Lower Greensand, no stygobites were found in this geology. In contrast, copepods occurred at most sites and usually at high densities (Fig. 3.3a). The Lower Greensand harboured several freshwater and terrestrial taxa, with oligochaetes (Table 3.4) and collembolans (Table 3.5) being most abundant and widespread.

Overall, *M. leruthi* was the most widespread (4 sites) and abundant stygobite species, while *N. aquilex* (3 sites) and *N. fontanus* (1 site) were rarer. Other relatively common (e.g. *N. kochianus*, *C. subterraneus*) or rare (e.g. *N. glenniei*, *A. stammeri*) stygobites known to occur in England and Wales were not found in the sampled geologies. Copepods were ubiquitous, occurring at high abundances in all 4 sampled geologies (Fig. 3.3a).

Throughout the study area, stygobites were much rarer than copepods, other freshwater taxa (see methods for included taxa) and terrestrial taxa (Tables 3.4, 3.5). Stygobites only occurred in 26.8 % of the sampling sites, while copepods (51.1 %), other aquatic taxa (82 %) and terrestrial taxa (72 %) were found much more frequently (Table 3.5). Furthermore, the occurrence of faunal groups differed between geologies. Stygobites were found more frequently in limestones (60 % in Jurassic Limestone) than in sandstones (none in Lower Greensand) (Table 3.4). Copepods were found at 25 % of sites in Magnesian Limestone and at 80 % of sites in Jurassic Limestone. Other freshwater taxa and terrestrial taxa occupied a large proportion of sites in the sampled geologies (50 – 100 %) (Tables 3.4, 3.5).

Freshwater and terrestrial taxa were much more abundant than stygobites in all four geologies (Fig. 3.4; Tables 3.4, 3.5). Copepoda and Oligochaeta were the most widespread and abundant freshwater taxa occurring in all geologies, while Cladocera and Trichoptera larvae were relatively rare (Table 3.4). Terrestrial taxa were found in all geologies, ranging from widespread (Arenae and Collembola) to rare (Pseudoscorpiones, Coleoptera and Hymenoptera) (Table 3.5).

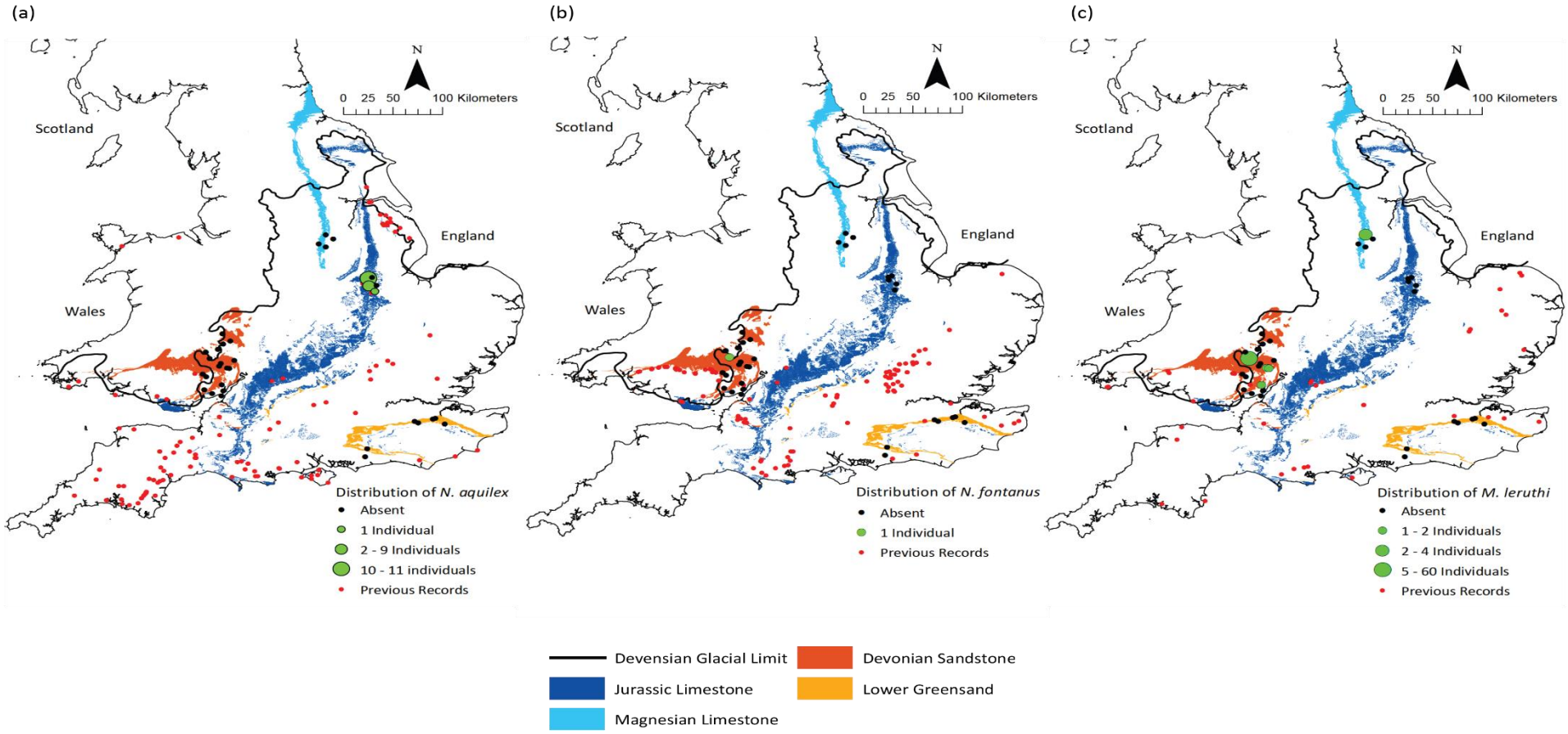

Fig. 3.2: Distribution maps of the stygobite species (a) *N. aquilex*, (b) *N. fontanus* and (c) *M. leruthi* in the sampled geologies. Also shown are their previous records and the southern Devensian glaciation limit. Green circles show relative species abundances at sites where they were found.

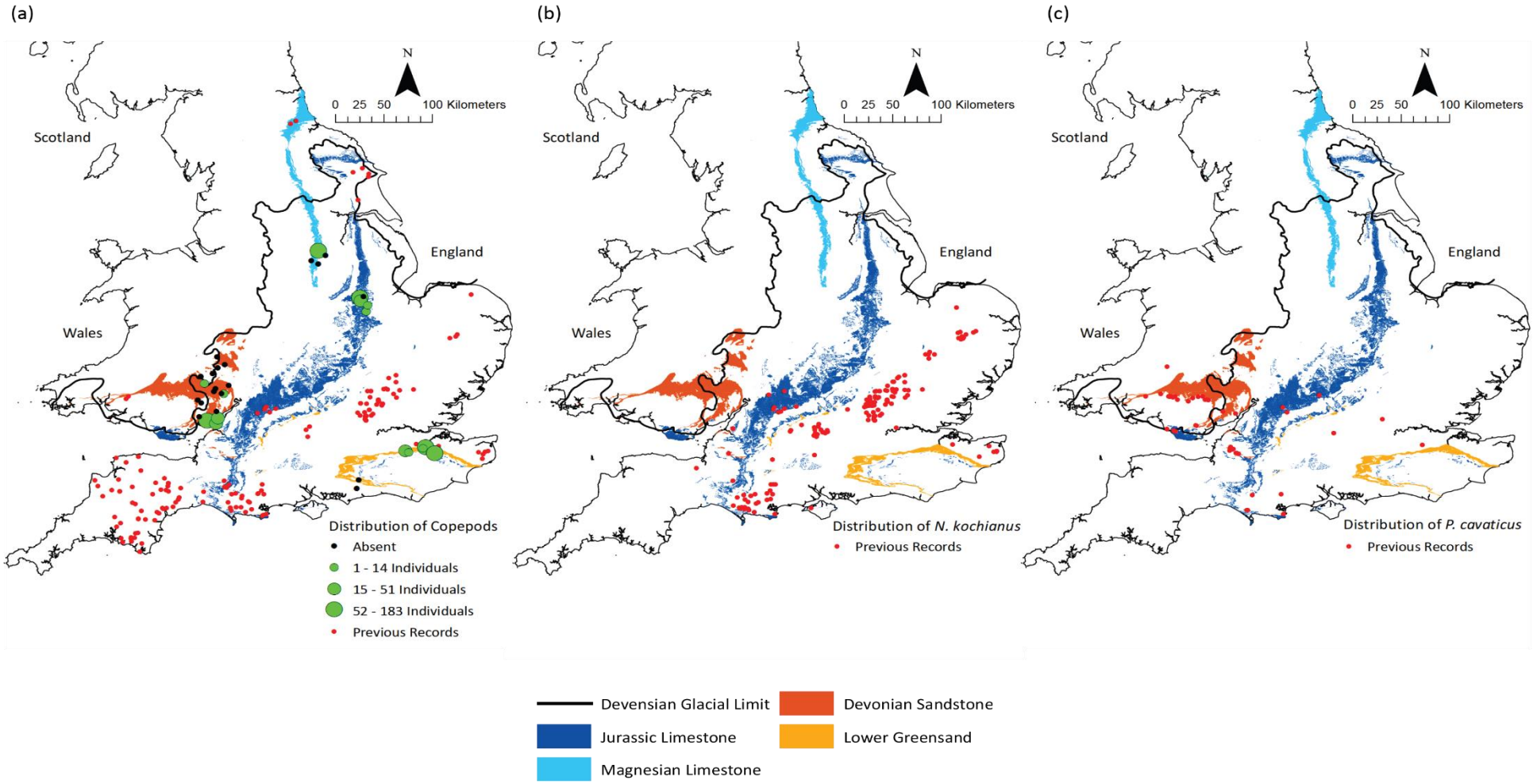

Fig. 3.3: Distribution maps of (a) copepods, (b) *N. kochianus* and (c) *P. cavaticus* in the sampled geologies. Also shown are their previous records and the southern Devensian glaciation limit. Green circles show relative species abundances at sites where they were found.

Table 3.3: Overview of the four areas sampled indicating the stygobite species present, the species expected based on currently known distributions, but absent; and whether absent species are found in similar geologies elsewhere in the UK.

Geology	Species Present (No. Sites)	Species expected based on range, but absent	Present in similar geologies elsewhere in the UK
Devonian Sandstone	*M. leruthi* (4 sites), *N. fontanus* (1 site)	*N. aquilex*, *P. cavaticus*	*N. aquilex* present in fractured sandstone in Devon / Dorset; *P. cavaticus* not present in fractured sandstone
Lower Greensand	None	*C. subterraneus, M. leruthi, N. aquilex N. fontanus, N. kochianus, P. cavaticus*	*N. aquilex* and *M. leruthi* present in intergranular sandstone in Devon / Dorset; the others have not been found in intergranular sandstone
Jurassic Limestone (northern outcrop)	*N. aquilex* (3 sites)	*C. subterraneus, N. fontanus, N. kochianus, M. leruthi*	Species present in southern outcrop of Jurassic Limestone and other karstic geologies
Magnesian Limestone (southern outcrop)	*M. leruthi* (1 site)	*N. aquilex, C. subterraneus*	*N. aquilex* present in many other karstic geologies

Table 3.4: Summary of the number of sampling sites (N) and the occurrence as proportion of sampled sites (%) and abundance (Ab) of stygobites and other freshwater taxa in the four sampled geologies. Also given is the mean abundance per site (M).

		Stygobites						Freshwater taxa													
Geology	**N**	***N. aquilex***		***N. fontanus***		***M. leruthi***		**Acari**		**Cladocera**		**Isopods**		**Amphipods (excl. Stygobites)**		**Copepoda**		**Trichoptera (larvae)**		**Oligochaeta**	
		%	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**
Devonian Sandstone	18	-	-	5.6	3 (0.2)	22.2	64 (3.6)	27.8	18 (1)	11.1	38 (2.1)	16.7	51 (2.8)	11.1	34 (1.9)	27.8	234 (13)	11.1	4 (0.2)	11.1	152 (8.4)
Lower Greensand	7	-	-	-	-	-	-	28.6	3 (0.4)	14.3	2 (0.3)	14.3	3 (0.4)	-	-	71.4	228 (32.6)	-	-	57.1	18 (2.6)
Jurassic Limestone	5	60	21 (4)	-	-	-	-	60	14 (2.8)	-	-	20	1 (0.2)	20	1 (0.2)	80	181 (36.2)	20	2 (0.4)	20	1 (0.2)
Magnesian Limestone	4	-	-	-	-	25	4 (1)	-	-	-	-	-	-	-	-	25	183 (45.8)	-	-	25	14 (3.5)

Table 3.5: Summary of the number of sampling sites (N) and the occurrence as proportion of sampled sites (%) and abundance (Ab) of terrestrial taxa and broad faunal groups in the four sampled geologies. Also given is the mean abundance per site (M).

		Terrestrial taxa														Broad groups		
Geology	**N**	**Arenae**		**Coleoptera**		**Collembola**		**Diptera**		**Hymenoptera**		**Orthoptera**		**Pseudoscorp**		**Stygobites**	**Other Freshw. Taxa**	**Terrestrial Taxa**
		%	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**Ab (M)**	**%**	**%**	**%**
Devonian Sandstone	18	5.6	1 (0.1)	16.7	5 (0.3)	22.2	12 (0.7)	33.3	7 (0.4)	22.2	6 (0.3)	5.6	1 (0.1)	-	-	22.2	77.8	72.2
Lower Greensand	7	28.6	3 (0.4)	-	-	57.1	46 (6.6)	-	-	-	-	42.9	6 (0.9)	14.3	1 (0.1)	-	100	85.7
Jurassic Limestone	5	40	2 (0.4)	40	10 (2)	80	15 (3)	60	12 (2.4)	20	1 (0.2)	60	5 (1)	-	-	60	100	80
Magnesian Limestone	4	25	2 (0.5)	-	-	50	2 (0.5)	-	-	-	-	-	-	-	-	25	50	50
Mean Total		-	-	-	-	-	-	-	-	-	-	-	-	-	-	26.8	82	72

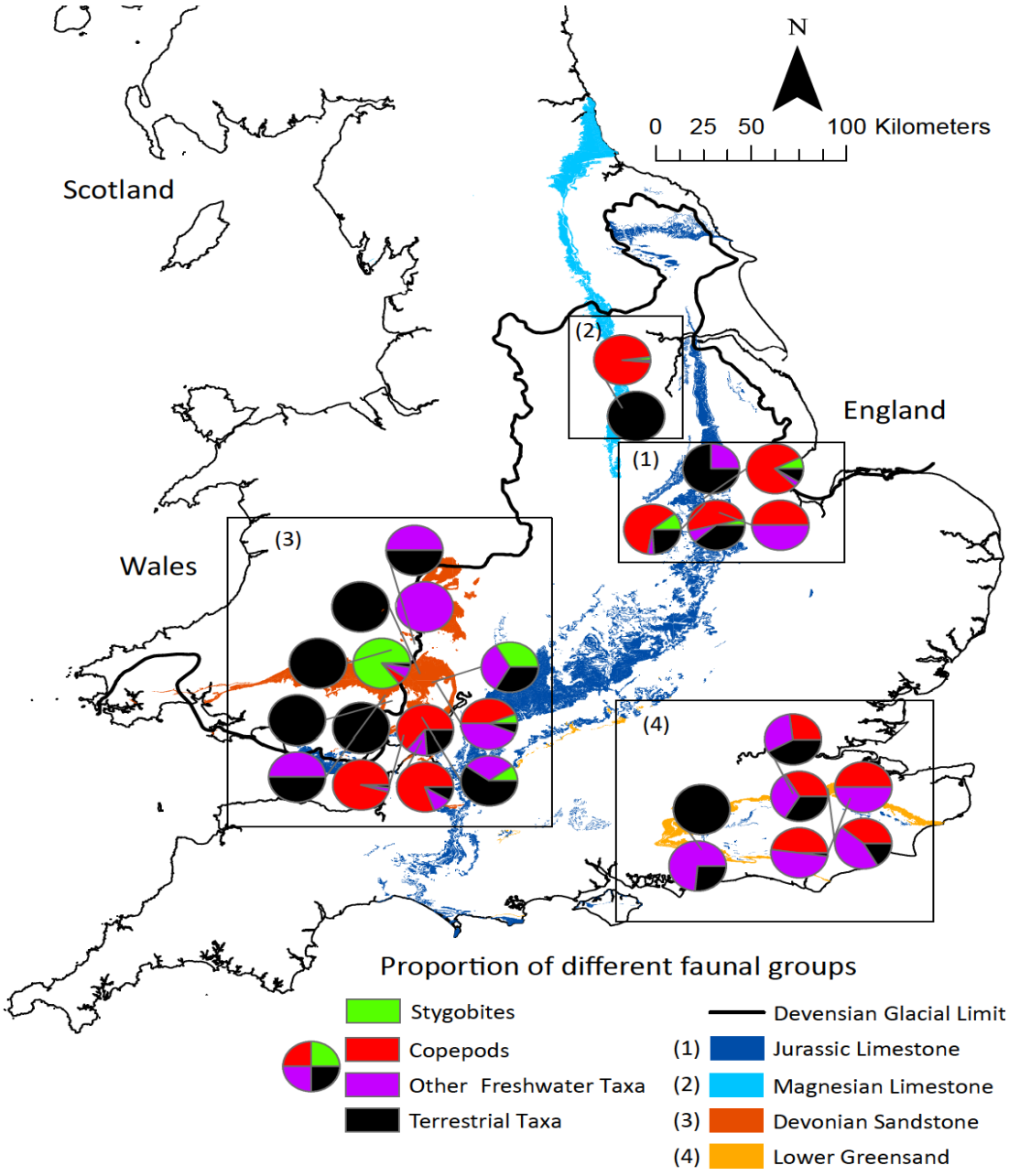

Fig. 3.4: Map showing the proportion of stygobite and freshwater taxa in the four sampled geologies in England and Wales.

3.4.2 Biomass trends in geologies

The total biomass (corrected for sample size) present in the geologies differed considerably, with the highest total biomass being found in Jurassic Limestone (10,922 μg C) followed by the Devonian Sandstone (1724 μg C). The Magnesian Limestone harboured the lowest total biomass (173 μg C) (Fig. 3.5, Table 3.6). In all of the sampled geologies stygobites only made up a small amount of the cumulative biomass (Fig. 3.5, Table 3.6). Stygobites contributed more to the total biomass in limestones than in sandstones, with the highest proportion in the Magnesian Limestone (15.1 %) (Fig. 3.5, Table 3.6). Copepods, other aquatic freshwater taxa (up to 85.7 %) and terrestrial taxa (up to 89.8 %) all contributed much larger proportions to the cumulative biomass in all four geologies (Table 3.6). Furthermore, the terrestrial biomass did not significantly differ between open and sealed sampling sites (data not shown).

Table 3.6: Summary of biomass data for different faunal groups in the four geologies corrected by the number of sampling sites. Shown is the total biomass, and the % contribution of stygobites, copepods, other freshwater taxa and terrestrial taxa.

Geology	Total Biomass (µg C)	% contribution to total biomass by			
		Stygobites	Copepods	Other Freshw. Taxa	Terrestrial Taxa
Devonian Sandstone	1724	5.5	4.8	85.7	4.2
Jurassic Limestone	10922	6.9	1.8	1.5	89.8
Lower Greensand	532	0	29.7	24.6	45.8
Magnesian Limestone	173	15.1	39.3	0.03	45.5
Mean Total	**13351**	**6.9**	**18.9**	**13.7**	**46.3**

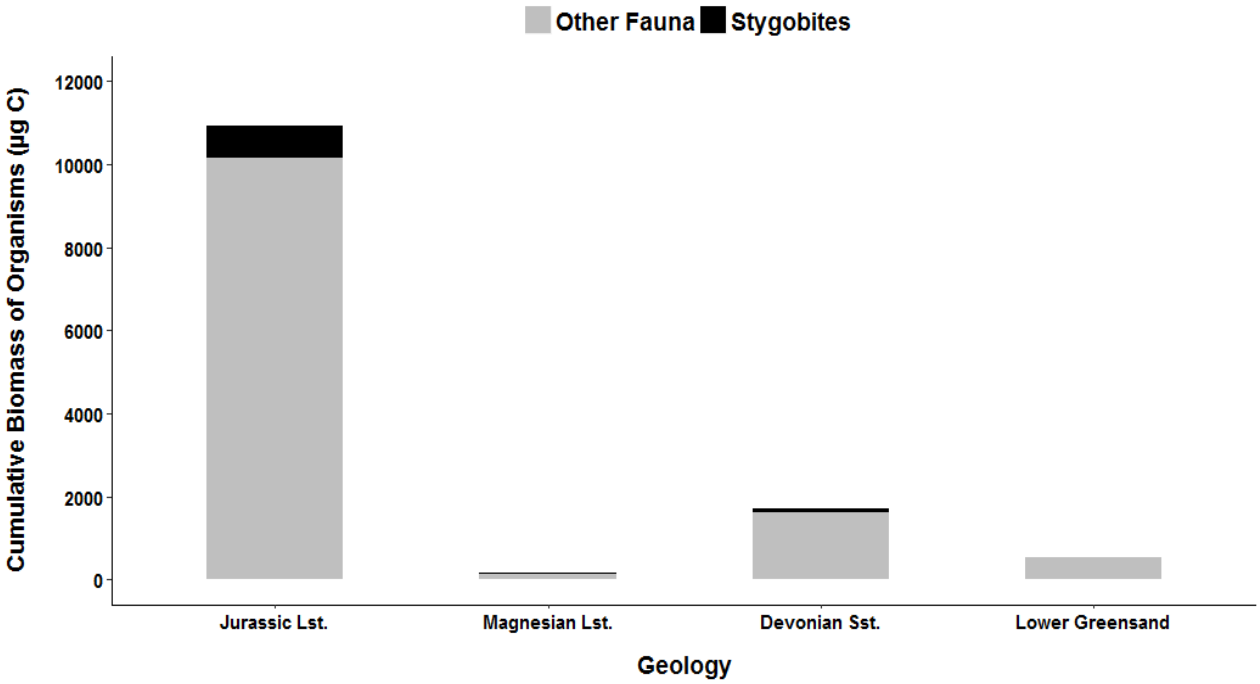

Fig. 3.5: Relative contribution of stygobites and other fauna to the cumulative biomass in µg carbon of the four sampled geologies. Bars are corrected for the number of samples in each geology.

3.4.3 Individual copepod biomass and abundance

Local site effects

The Kruskal-Wallis test indicated that copepod biomass varied significantly between individual sampling sites (H_{14} = 345.64, P < 0.001) (Fig. 3.6). Within the same geology some sampling sites (e.g. St. Tewdrick, Essendine Bourne Road, Heath House) had highly variable copepod biomass, while others (Abby Dore, Old Well House) showed very little variability (Fig. 3.6). Due to the strong influence of individual sampling location and many individuals originating from the same site (being subject to the same environment), sampling site was fitted as a random factor in the following copepod biomass models.

Copepod abundance also varied considerably between individual sampling sites within the same geology (Fig. 3.6). In Jurassic Limestone copepods ranged between 1 and 116 individuals (mean: 36), in Devonian Sandstone between 4 and 146 individuals (mean: 13) and in Lower Greensand between 1 and 99 individuals (mean: 33). In the Magnesian Limestone 183 copepods only occurred at a single site (Fig. 3.6). All geologies had sites where copepods were completely absent (data not shown).

Overall, the influence of random local site effects was always significantly stronger than that of other environmental predictors in the linear mixed-effects models (Table 3.7). Environmental parameters explained between 3 % and 18 % of variance, while random site characteristics explained between 35 and 53 % of variance of copepod biomass. Furthermore, copepod abundance was only significantly influenced by physical cover, which is one such site-specific characteristic (see below).

Other environmental effects

Geology did not significantly influence copepod biomass and abundance (Tables 3.7 and 3.8; see Appendix 3.1 for biomass model assumption tests). However, there was a trend towards higher copepod biomass in Devonian Sandstone and Jurassic Limestone, and considerably lower biomass in Magnesian Limestone (Fig. 3.7). Furthermore, there was a trend towards higher copepod abundances in the limestones compared to sandstones (Fig. 3.7).

Higher copepod biomass was significantly predicted by the presence of superficial deposits ($B = 6.7$, $z = 12.3$, $P < 0.05$) (Table 3.7), compared to the absence of deposits. Most other environmental variables only weakly influenced copepod biomass (Tables 3.7), but there was a trend towards lower copepod biomass in sealed than in open sampling sites.

In wells, copepod abundance was significantly influenced by cover, being higher in open than in sealed sites ($H = 8.57$, $P < 0.01$) (Table 3.8, Appendix 3.2). Unlike biomass, copepod abundance was not affected by the presence of superficial deposits ($H = 0.16$, $P > 0.05$), but there was a near-significant negative correlation between copepod abundance and depth (Table 3.8, Appendix 3.2). No significant relationships between environmental predictors and copepod abundance were apparent for boreholes.

Table 3.7: Results of linear mixed effects models (with sampling site fitted as random predictor) testing the influence of abiotic predictors on copepod biomass. The r^2 of both predictors and random site effects are given for comparison.

Response Parameter	Predictor (r^2)	Comparison	Estimate	Std. Error	*P*	Site (r^2)
Copepod Biomass	~ Geology (0.1)	Dev. Sst.	6.9	2.4	< 0.05 *	**0.48 (48 %)**
		Jur. Lst.	- 2.8	3.7	> 0.05	
		Low. Gs.	- 3.6	3.4	> 0.05	
		Mag. Lst.	- 5.4	5.6	> 0.05	
	~ Site Type (0.03)	Borehole	0.2	4.3	> 0.05	**0.53 (53 %)**
		Well	2.8	2.7	> 0.05	
	~ Cover (0.04)	Open	6.5	1.8	< 0.01 **	**0.49 (49 %)**
		Sealed	- 3.6	2.6	> 0.05	
	~ Sup. Dep. (0.18)	Absent	2.6	1.3	> 0.05	**0.35 (35 %)**
		Present	**6.7**	**2.3**	**< 0.05 ***	
	~ Season (0.10)	Autumn	2.9	2.2	> 0.05	**0.44 (44 %)**
		Spring	4	3.1	> 0.05	
		Summer	1.2	3.5	> 0.05	
	~ Depth (0.15)	-	-0.1	0.1	> 0.05	**0.4 (40 %)**
	~ Dist. Surf. (0.03)	-	< 0.001	< 0.01	> 0.05	**0.5 (50 %)**

Table 3.8: The effect of abiotic predictors on copepod abundance in wells showing results for Kruskal-Wallis tests and Kendall's tau correlations (+ see Appendix 3.2 for figures).

Response Parameter	Kruskal-Wallis tests				Kendall's tau correlation		
	Predictor	Df	X^2 (K-W)	P	Predictor	tau	P
Copepod Abundance	Geology	1	0.29	> 0.05	Depth (+)	-0.43	> 0.05
	Cover (+)	**1**	**4.72**	**< 0.05 ***	Dist. Surf.	0.31	> 0.05
	Sup. Dep.	1	0.1	> 0.05	Dist. Glac.	-0.18	> 0.05
	Season	2	0.29	> 0.05	Water Table	-0.01	> 0.05

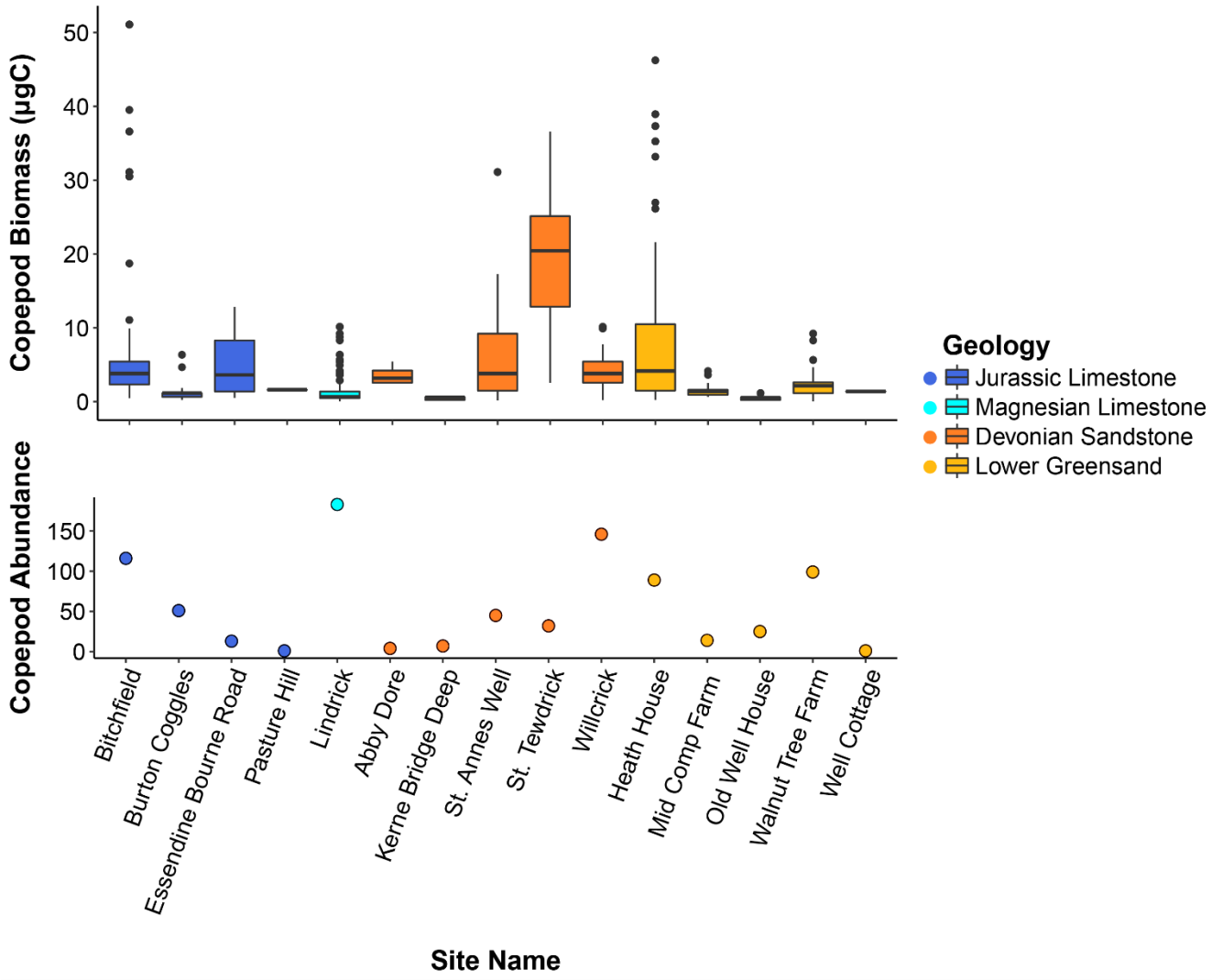

Fig. 3.6: Variation in copepod biomass (µg C) (top) and abundance (bottom) in individual sampling sites grouped according to geology.

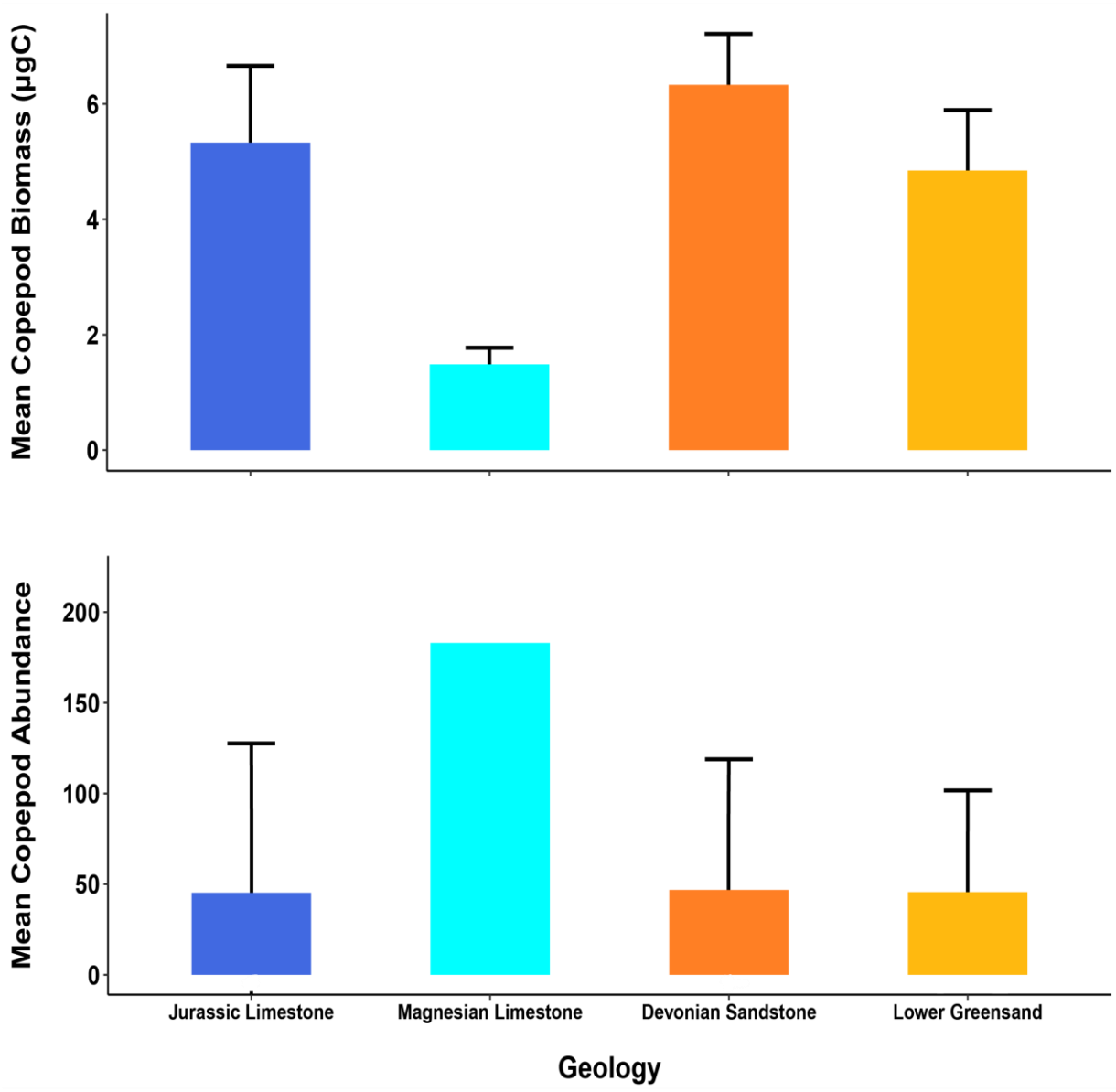

Fig. 3.7: Effects of geology on copepod biomass (µg C) (top) and copepod abundance (bottom). Standard error is not available for copepod abundance in Magnesian Limestone, because they were only found at a single site.

3.5 Discussion

3.5.1 Stygobite distributions in the four geologies

Across the four geologies, stygobites occurred more frequently in the limestones (25 – 60 %) compared to the sandstones (0 – 22.2 %). With only one species, the stygobite diversity in the Magnesian Limestone was very low, which could be partly related to the low sampling effort of only nine sites. This study provides the most northerly record of *M. leruthi* and the first evidence for stygobites in the Magnesian Limestone. Previously thought to be endemic to mainland Europe, and probably often overlooked due to its small size, *M. leruthi* has been discovered in various geologies since 2006 (Knight & Gledhill, 2010; Maurice et al., 2015). Overall, the Magnesian Limestone has relatively high transmissivity (233 m^2/d; Allen et al., 1997) and was assessed as being a good habitat for groundwater fauna (Weitowitz et al., submitted). The absence of most species is therefore likely due to be due to controls other than geology. Stygobites were absent at sites in the northern outcrop of the Magnesian Limestone north of the Devensian glacial limit, indicating the potential importance of past glaciations. Extirpations due to past glaciations are thought to be influential, because of the slow recolonisation rates of stygobites (Danielopol, 1991; Eberhard et al., 2009; Gibert et al., 2009; Robertson et al., 2009). In the southern outcrop of the Magnesian Limestone the low stygobite diversity may be reinforced by adjacent low-permeability geologies (e.g. mudstones), acting as barriers to dispersal (Hahn & Fuchs, 2009; Johns et al., 2015). Many species including N. *kochianus*, *N. fontanus*, *P. cavaticus* and *N. glenniei* are widespread in karstic habitats in southern England and Wales, but do not appear in similar habitats in the north

(Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015), indicating this may remain outside their geographical range.

Previous sampling determined high species diversity (*N. aquilex*, *N. fontanus*, *N. kochianus* and *M. leruthi*) and abundance in the southern outcrop of Jurassic Limestone (HCRS, 2016). The high suitability of Jurassic Limestone as a stygobite habitat was also evident in the northern outcrop, where *N. aquilex* was found at 60 % of sites. The mean transmissivity of the moderately karstic Jurassic Limestone is 665 m^2/d (Allen et al., 1997) and it was found to provide highly suitable habitat in a previous assessment (Weitowitz et al., submitted). It is likely to be a good habitat due to its karstic properties that provide abundant living space and high connectivity, in turn promoting nutrient influx and species dispersal (Culver & Pipan, 2011). Overall, the stygobite community in the Jurassic Limestone provides further evidence that karstic rocks are highly important habitats, harbouring diverse stygobite assemblages both in the UK (Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015) and across the world (Culver & Sket, 2000; Christman et al., 2005; Deharveng et al., 2009; Gibert et al., 2009; Hahn & Fuchs, 2009).

Despite suitable habitat conditions throughout the Jurassic Limestone aquifer and the high frequency of occurrence of *N. aquilex*, this was the only species detected in its northern outcrop. This was surprising because the Jurassic Limestone is highly transmissive (Allen et al., 1997) and unconfined, and would be expected to facilitate species migrations from the biodiverse southern outcrop. Therefore, similar to the Magnesian Limestone, species presence in this aquifer seems not to be solely determined by habitat quality. For some species that have been documented in other geologies in northern England (e.g. *A. stammeri*, *M. leruthi* and *C. subterraneus*) the observed absence

may be due to insufficient sampling (5 sites). For other species including *N. kochianus* (very common in the southern outcrop), *N. fontanus* and *P. cavaticus*, the northern outcrop appears to remain outside their ranges. The proximity of the northern Jurassic Limestone to the Devensian glacial limit, probably means that habitat conditions (e.g. water temperature, pressure) deteriorated considerably during the glaciation, resulting in local extinctions. Stygobites have not dispersed through a single, connected aquifer since the last glaciation, which is likely to contribute to the long-term static distribution of faunal lineages (see McInerney et al., 2014). In the Jurassic Limestone, *N. aquilex* appears to have the most northerly distribution, suggesting that it may disperse faster than other species or may have survived the glaciation in subsurface refugia. The fact that *N. aquilex* is frequently found in hyporheic zones (HCRS, 2016), suggests it may utilise hyporheic corridors for more efficient dispersal (chapter four, Ward & Palmer, 1994).

Two species, *N. fontanus* and *M. leruthi*, were documented in the Devonian Sandstone. Their presence in directly adjacent geologies, such as the Carboniferous Limestone to the south, indicates that some connectivity and dispersal pathways between these geological strata may be present now or may have existed in the past. However, stygobites only occurred at 22 % of sites, suggesting that Devonian Sandstone is a reasonable habitat but not as favourable as the limestones or the Chalk, where stygobites occurred at 67 % of sites (Maurice et al., 2015). Species that do occur in both the Devonian Sandstone and the Chalk also have lower frequencies of occurrence in the sandstone (*N. fontanus*: 5.6 % cf. 38 %, *M. leruthi*: 22.2 % cf. 31 %). Although the Devonian Sandstone was adequately sampled, some species common in southern England (e.g. *N. aquilex* and *N. kochianus*) were absent. The most likely explanation for the low frequency of occurrence of stygobites may be that poor quality habitat patches

are often present in the Devonian Sanstone. These are likely to have low transmissivity (mean of 51 m^2/d cf. mean of 1600 m^2/d in the Dorset Chalk; Allen et al., 1997; Jones et al., 2000) and fracture density, and frequent mudstone impurities (also see Weitowitz et al., submitted), possibly restricting fauna to a few suitable sites. Some species that are common in the Chalk of eastern England (e.g. *N. kochianus*) may be prevented from colonising the Devonian Sandstone by poor habitats (mudstones, siltstones), separating their main habitat from the sandstone (see chapter two). Furthermore, some larger stygobite species may be excluded from small fractures (sometimes approx. 1 cm; Hooker et al., 2011; Inigo et al., 2012) due to their body size. Other studies have also found depauperate stygobite assemblages in fractured sandstones (Hahn & Fuchs, 2009; Robertson et al., 2009; Johns et al., 2015), although some sandstones in Australia harbour high species diversity (Humphreys, 2008). Overall, the invertebrate ecology of the Devonian Sandstone appears to be determined by a combination of geological and dispersal constraints.

Stygobites were absent from samples in the Lower Greensand, although many of the abiotic parameters should be sufficient to sustain stygofauna (Weitowitz et al., submitted). For example, the relatively high transmissivity of 270 m^2/d maintains moderate-high levels of dissolved oxygen, dissolved organic carbon and calcium (Allen et al., 1997; Shand et al., 2003a). Furthermore, dispersal should not be a limiting factor, because the Lower Greensand lies within the distribution range of multiple species (*N. aquilex*, N. *kochianus*, *N. fontanus*, *C. subterraneus* and *P. cavaticus*) in the adjacent Chalk (Maurice et al., 2015). Stygobites are probably absent because this aquifer consists largely of unconsolidated sands with small pore spaces (maximum diameter of approx. 1.3 mm in coarser sands, Cook, 2002) that allow high transmissivities, but are too small for the

larger metazoans to live in. Copepods, which occurred at 71.4 % of sites (average body length of 0.7 mm from 228 individuals in this geology) in the Lower Greensand, are probably just small enough to live in the diminutive pore spaces. The Lower Greensand also contains clays and, in consolidated sections, mudstones (Allen et al., 1997), which are unlikely to be colonised by stygobites. Porous habitats, such as quaternary deposits (e.g. Johns et al., 2015) and river alluvium (e.g. Hahn & Fuchs, 2009), harboured diverse and abundant stygobite communities. However, these habitats consist of gravels and pebbles with large pore spaces (Dole-Olivier et al., 2009a), which are not reflective of the conditions in the Lower Greensand.

Some stygobite species were not detected in any of the sampled geologies because they are either very rare (*A. stammeri*) or occur predominantly in caves of south-western England and southern Wales (*P. cavaticus*). For example, only a few records of *A. stammeri* exist in the UK, indicating that the present sampling effort was far too low to reliably detect this species. As in previous studies (Dole-Olivier et al., 2009a; Johns et al., 2015), stygobites were only found at 26.8 % of sites, and occurred at lower abundances than other freshwater fauna, such as copepods, gammarids and oligochaetes.

3.5.2 Overall biomass pattern

Overall, sites in the four sampled geologies clearly contained different amounts of carbon, with Jurassic Limestone containing the highest and Magnesian Limestone having the lowest total carbon. A major proportion of biomass in sampling sites is made up of terrestrial taxa that do not naturally occur in freshwater. Terrestrial carbon may be essential in sustaining groundwater assemblages by providing additional carbon to the

base of groundwater food webs, which are known to be extremely nutrient limited (Gibert et al., 1994; Hahn, 2006; Humphreys, 2006; Boulton et al., 2008; Schmidt & Hahn, 2012). The high levels of terrestrial carbon could be the consequence of site-specific (e.g. site cover) or aquifer-specific (e.g. lithology) features. The main source of terrestrial carbon may be organismal carbon that falls directly into a site, which may be exacerbated by the ecology sampling and other local activities at the sites. For example, in the Devonian Sandstone and Lower Greensand, many sites had no physical cover, increasing the likelihood of terrestrial material falling in. Other factors, such as site diameter and surrounding land use are also likely to affect the amount of terrestrial carbon. For example, sites with larger diameters in grassland may be expected to receive more terrestrial carbon than sites with smaller diameters in surroundings made of concrete.

However, in karstic geologies it is also possible that some of the terrestrial biomass is carried underground in sinking streams (Culver & Pipan, 2009). Several stream sinks are known in the Jurassic Limestone (e.g. Foley et al., 2012; Atkinson, 2015) and may contribute to its large pool of terrestrial biomass. However, this is relatively unlikely because karstic features in Jurassic Limestone are less well developed than in other geologies (e.g. Carboniferous Limestone) and boreholes / wells often do not intercept the main conduits of aquifers.

Stygobites (0 – 15.1 %) and copepods (1.8 – 39.3 %) contributed only a small proportion to the total biomass in the sampled geologies, compared to other freshwater taxa (0.03 – 85.7 %) and terrestrial taxa (4.2 – 89.8 %). However, in relative terms stygobites contributed most biomass in the Magnesian and Jurassic Limestones. This was the case despite all sampling sites in the limestones being sealed. It was expected that

open sites would receive higher nutrient input (see copepod discussion), which would also be reflected in increased stygobite biomass. This indicates that the higher stygobite biomass in limestones most likely reflects their preference of such habitats and is a genuine aquifer-specific effect. This corroborates previous studies, which have also found the highest stygobite abundances in karstic rocks (Christman et al., 2005; Hahn & Fuchs, 2009; Robertson et al., 2009). Overall, the higher relative stygobite biomass in the limestones compared to the sandstones is most likely due to their higher permeability, greater surface connectivity and higher nutrient concentrations (Dole-Olivier et al., 2009a; Weitowitz et al., submitted).

Although the investigated taxa are important components of groundwater communities, some types of organisms were not considered in this study. Groundwater biofilms contain up to 40 % of the earth's prokaryotic biomass (Griebler & Lueders, 2009) and unicellular protozoans, which contribute significantly to the biomass of freshwater ecosystems (Reiss & Schmid-Araya, 2010). These biofilms are basal ecosystem components mediating the energy flux to higher trophic levels (Boulton et al., 2008) and should be considered in future studies approximating and comparing biomass between geologies.

One of the main criticisms of groundwater studies is that boreholes and wells, our only access points to groundwater ecosystems, are artificial habitats receiving high inputs of resources, and are therefore not representative of the surrounding aquifers (Hahn & Matzke, 2005). Indeed, a study using targeted packer pump testing showed that bacterial and invertebrate abundances were significantly higher in boreholes than in the surrounding aquifer, with non-linear abundance declines with distance from the borehole

(Sorensen et al., 2013). Individual biomass (related to resources) and total biomass (related to abundance) are measures that are inevitably influenced by this so-called 'borehole effect'. Nevertheless, net sampling gives a useful overview of groundwater communities. For example, it was also determined that the taxonomic composition in boreholes is comparable to the surrounding aquifers (Hahn & Matzke, 2005). Biomass samples are therefore useful to compare geologies, although future studies should select sampling sites with similar characteristics (e.g. physical cover, depth, diameter) to minimise data bias.

3.5.3 Influences on copepod biomass and abundance

Because stygobites were rare in the study area, copepods were used as the faunal group to investigate environmental controls on biomass and abundance in groundwater ecosystems. Copepods are frequent stygophiles in groundwater and several UK species show high subterranean affinity (Proudlove et al., 2003). However, the small number of sampling sites available for analysis, highlight that any conclusions should be drawn with caution.

In the statistical models based on 826 copepod measurements, differences between sampling sites accounted for a far greater proportion of variance (35 – 50 %) in copepod biomass than other environmental factor, such as geology (10 %). Figure 3.5 shows that the mean biomass varies considerably between sites and that all geologies have sites with highly variable and highly constrained copepod biomass. Between-site differences have also been shown to influence faunal parameters in other groundwater

studies (e.g. Eberhard et al., 2009; Hancock & Boulton, 2009), where stygobite abundances varied from a few to 300 stygobites between sites in the same geology.

It is likely that the most important controls on biomass and abundance patterns are non-aquifer site-specific factors, such as the presence or absence of physical cover. Copepod abundance was significantly lower in sealed than in open wells, which often contained large amounts of visible particulate organic carbon. Open sites may have increased nutrient concentrations compared to sealed sites, allowing these to sustain higher numbers of copepods. Bottom-up stimulatory effects of nutrients and detritus on invertebrates have been observed in both surface water and groundwater studies (e.g. Rosemond et al., 2001; Greenwood et al., 2007; Gruner et al., 2008; Griebler et al., 2010), and may be very important for the nutrient-limited groundwater communities. Previous research has shown that cross-ecosystem energy fluxes often provide a significant contribution to the base of food webs in the recipient communities (Polis et al., 2004; Greig et al., 2012). However, biomass and abundance differences between sites may also reflect small-scale variability of habitat patches in a heterogeneous environment (Dole-Olivier et al., 2009b; Larned, 2012; Weitowitz et al., submitted).

These unexplained between-site differences illustrate the need for considering site characteristics and geology in higher detail and at smaller spatial scales in sampling designs, to enhance the explanatory power of studies. As shown above for copepod abundance, one such characteristic may be physical site cover but many other factors, including the material and slotting of borehole casing, frequency of use and purpose of site, and the surrounding land use may be equally important. One, albeit relatively

expensive, way of avoiding this would be to create a network of custom-drilled boreholes with consistent specifications.

Geology did not appear to control copepod biomass and abundance in this small dataset, although previous studies in the Balkans found that limestones harbour very high copepod abundances (Pipan et al., 2006; Pipan & Culver, 2007; Galassi et al., 2009). Determining the effect of geology was difficult because only few sites with very different environmental characteristics were sampled in the four geologies. Packer pumping tests may be a more accurate approach to assess geological controls, because this technique allows the targeted sampling of aquifer water (see Sorensen et al., 2013) and removes the bias introduced by site characteristics.

One interesting pattern that emerged was that copepods had significantly higher biomass in sites covered by superficial deposits compared to sites without. In the study area superficial deposits are mainly alluvium or river terrace deposits, which are very permeable strata (Shand et al., 2003b; British Geological Survey, 2006) and potentially provide good habitats with high oxygen and nutrient concentrations. It is possible that the higher copepod biomass beneath superficial deposits reflects species-specific habitat preferences for interstitial gravels and sands (Galassi et al., 2009), with larger, possibly epigean copepod species originating from the deposits and migrating into the underlying bedrock. Copepod assemblages in superficial habitats were dominated by large-bodies copepod taxa in a previous study (Di Lorenzo et al., 2013). Furthermore, aquatic habitats are often characterised by a vertical zonation of copepods with oxygen and nutrient gradients, with different species dominating at particular depths (Wishner et al., 2008).

Overall, most investigated environmental parameters did not explain much of the variance in copepod biomass or abundance. One of the most important reasons for this is probably the low number of samples, which is mainly because access to groundwater habitats is limited to privately or government owned pre-existing boreholes and wells (Ruffo & Stoch, 2005; Ferreira et al., 2007; Dole-Olivier et al., 2009b). Furthermore, relating groundwater communities to abiotic variables is often difficult due to the heterogeneous environment and the complexity of interacting factors (see Dole-Olivier et al., 2009a,b; Johns et al., 2015). Therefore, a much larger dataset with higher sampling replication and repeated sampling visits is needed to better understand the controls of faunal biomass in groundwater. The importance of biomass as a measure of the functionality, dynamics and stability of surface ecosystems (MacArthur, 1955; McNaughton, 1985), and its known link with ecosystem services (e.g. pollutant degradation or transformation; Mattison et al., 2002, 2005) should provide a sufficient rationale for further investigations.

3.6 Conclusion

This paper provides the first ecological analysis of the four studied geologies, including the first samples in the Devonian Sandstone and Lower Greensand, and indicates that their relative importance as stygobite habitats clearly differs. The limestones generally harboured higher stygobite diversity and abundance than sandstones. Furthermore, the distribution controls seemed to differ between the geologies. In the Magnesian Limestone, only *M. leruthi* was found at a single site. In the Jurassic Limestone, harbouring high stygobite diversity (*N. kochianus*, *N. fontanus*, *N. aquilex* and *M. leruthi*)

in the southern outcrop, only *N. aquilex* was found in the northern outcrop. Since both limestones provide suitable habitat conditions throughout the entire aquifer, the faunal distributions are most likely not controlled ny geology, but by the glacial legacy and the low dispersal capacities of stygobites.

Devonian Sandstone harboured two species (*N. fontanus*, *M. leruthi*) at a low proportion of sites, while stygobites were absent from samples in the Lower Greensand. Because these habitats are considerably south of the Devensian glacial limit, and stygobite diversity and abundance is high in adjacent geologies, past glaciations and dispersal constraints are unlikely to be important distribution determinants in these habitats. Stygobite communities in the sandstones are most likely the result of geological controls, such as low fracture density and connectivity in Devonian Sandstone, and small pore spaces in the Lower Greensand. These patterns highlight that while geological habitat is clearly an important factor, large-scale processes (e.g. glaciations) often override geological controls. More extensive sampling, particularly in the Jurassic and Magnesian Limestone, is required to confirm these conclusions and their proposed underlying mechanisms.

The total biomass in the four investigated geologies varied considerably with Jurassic Limestone sustaining the highest total biomass followed by Devonian Sandstone. In all geologies except for Devonian Sandstone, the highest proportion of biomass was contributed by terrestrial organisms. This cross-ecosystem flux of biomass may be an important factor in sustaining resource-constrained groundwater communities. While representing only a fraction of the total biomass, stygobites contributed most carbon in

the Magnesian (15.1 %) and the Jurassic Limestone (6.9 %), indicating their preference of limestone over sandstone habitats.

The high proportion of copepod biomass explained by sampling site and the higher copepod abundances in open than in sealed wells, illustrate that site-specific factors may be a key determinant of faunal communities in groundwater. Geology exerted little influence on copepod parameters, highlighting that local-scale determinants (similar to large-scale processes) may also supersede the effects of geology. Overall, it is important to better integrate site characteristics and lithological detail at small spatial scales into high-replicate groundwater sampling designs. Biomass is one of the most widely used quantitative ecological descriptors in surface waters and may provide a useful additional tool for the assessment of individual and community-level responses to abiotic controls in groundwater.

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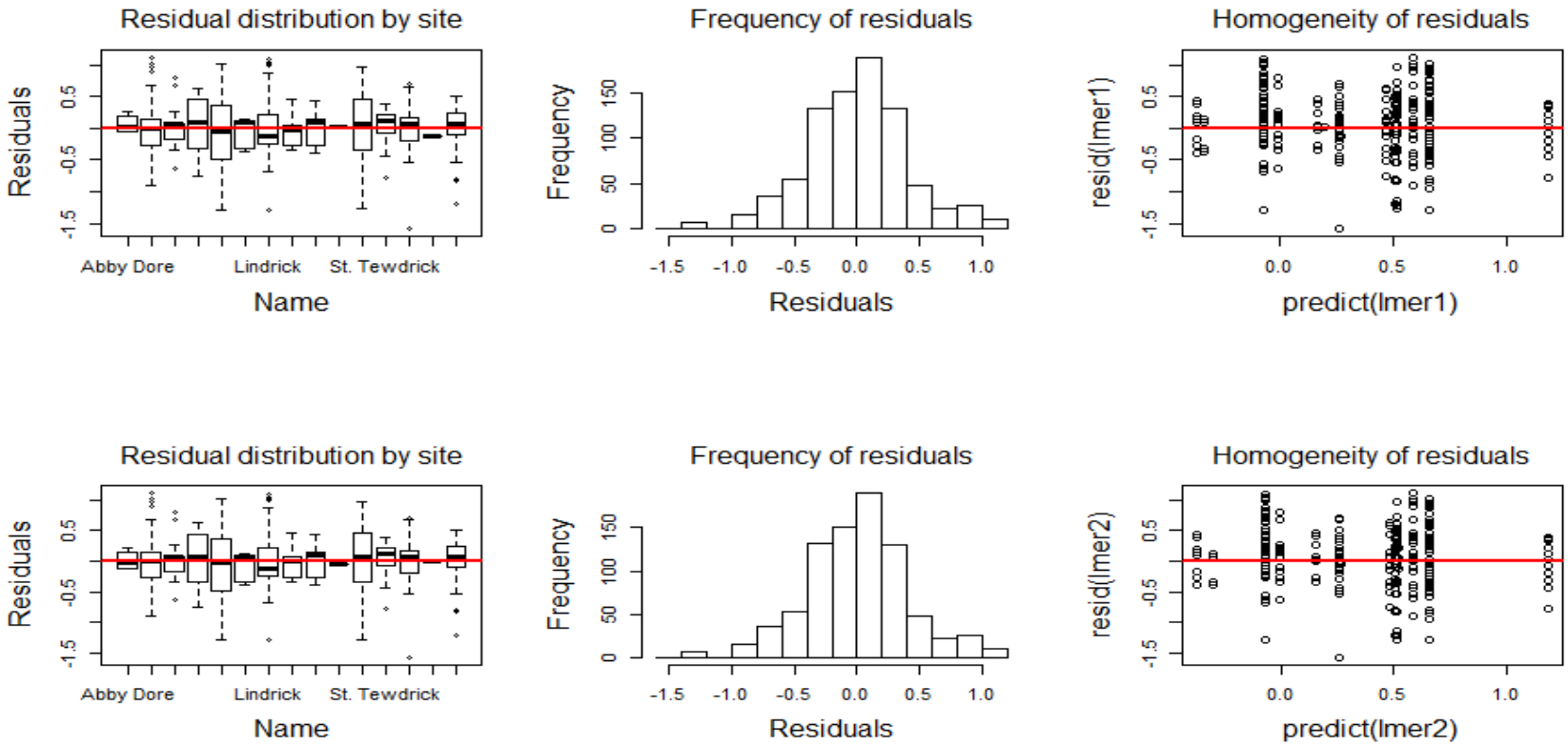
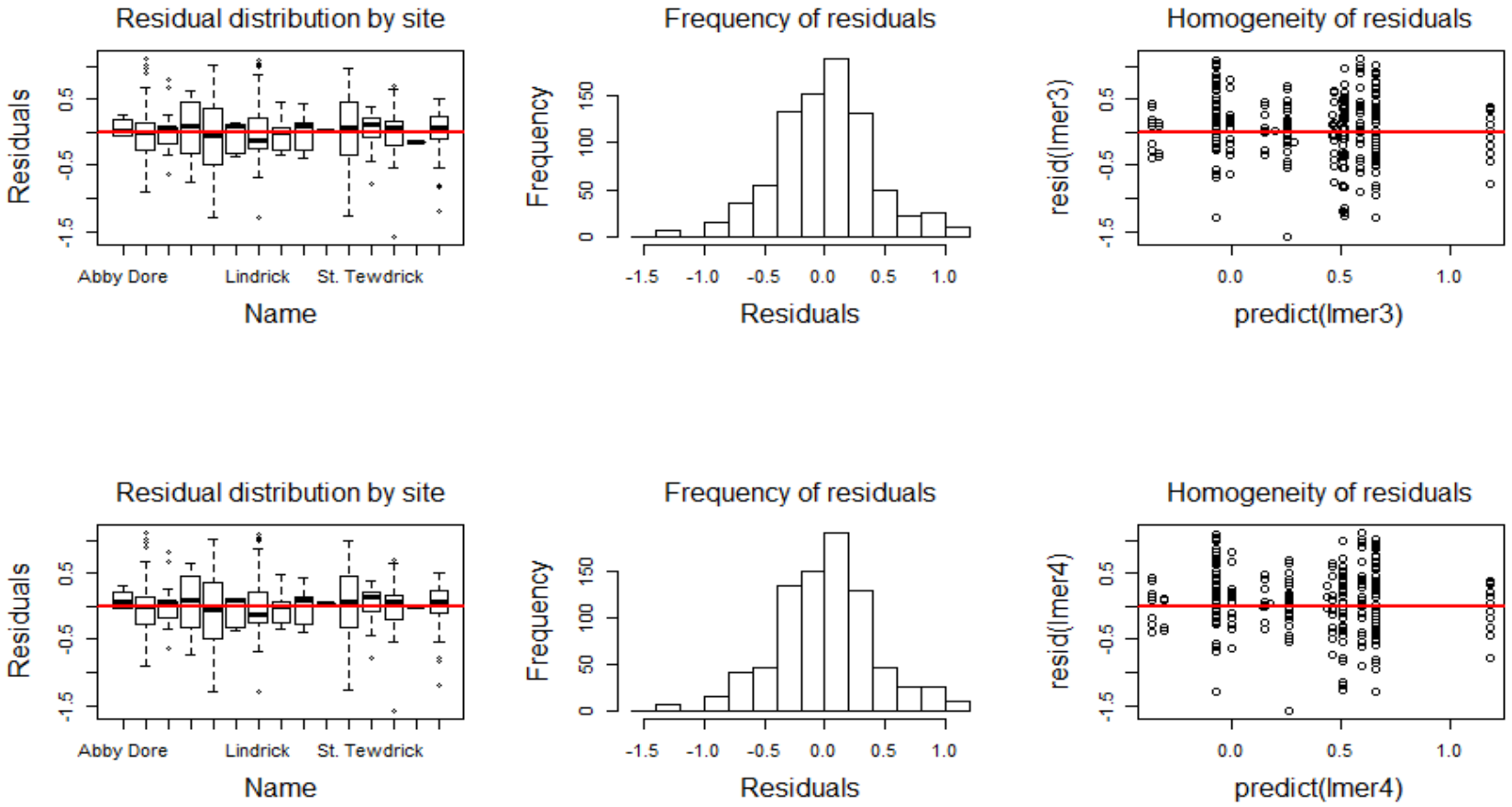
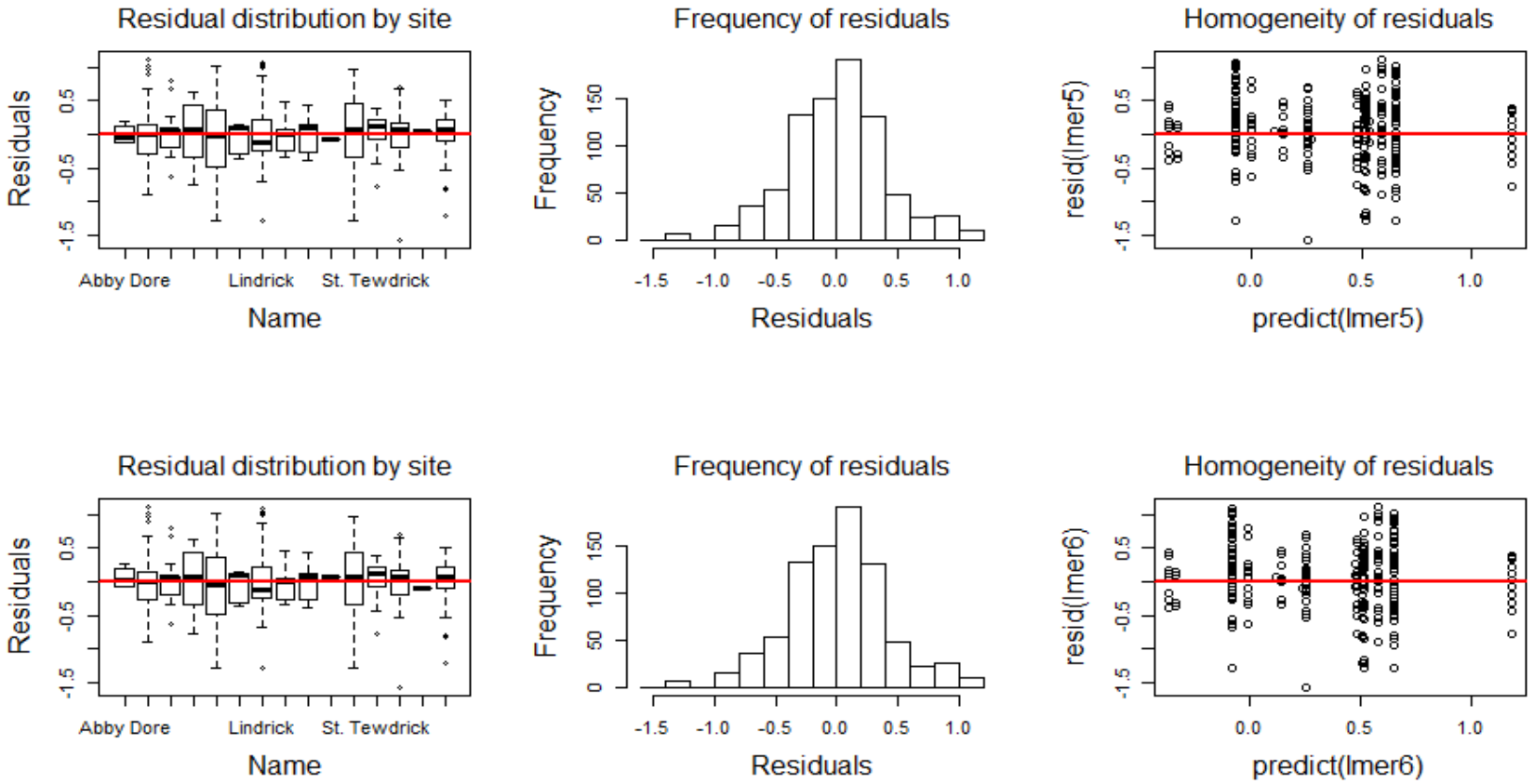
3.9 Appendices



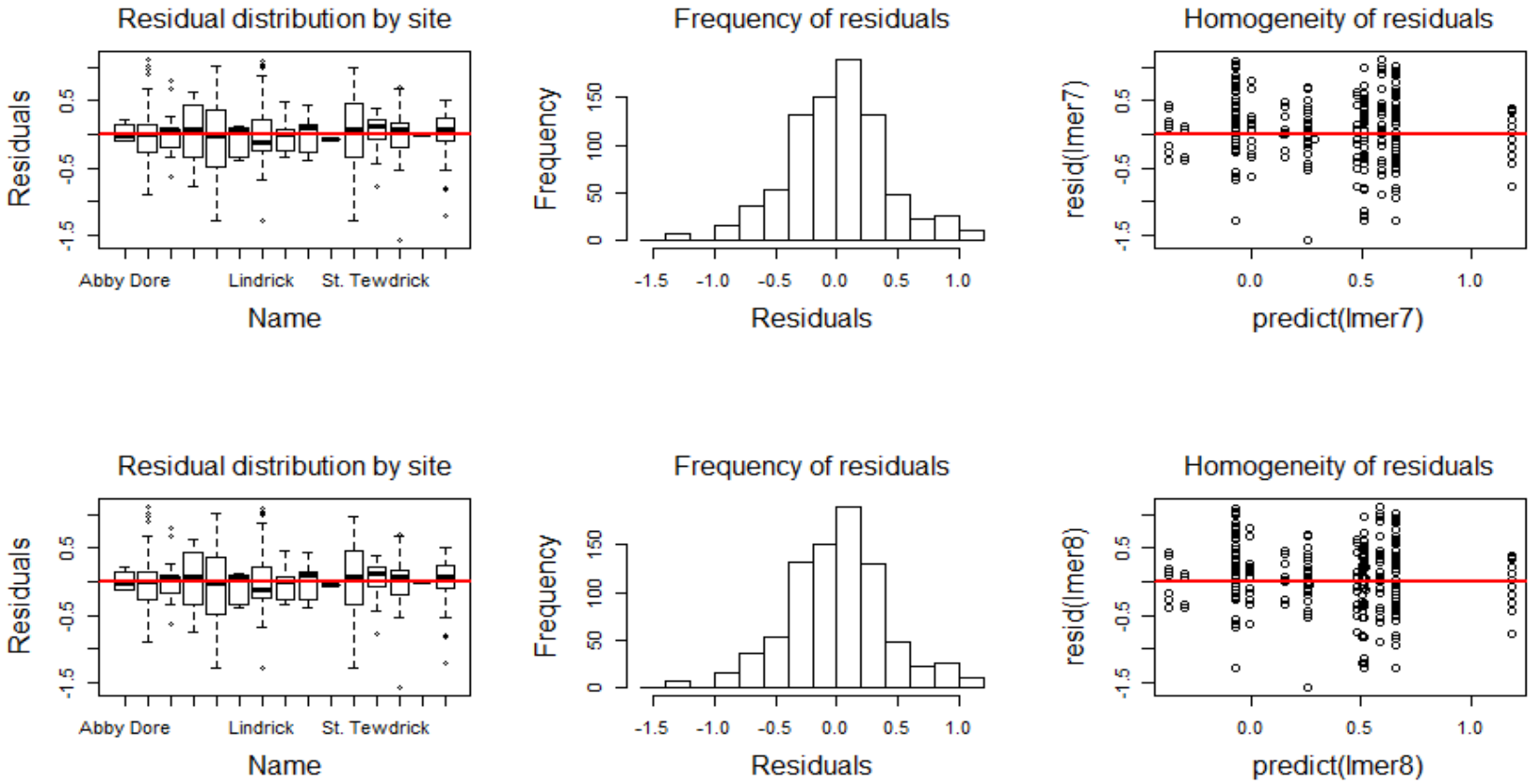
Appendix 3.1: Validation plots for copepod biomass linear mixed effects models showing the determinants site type (top row) and geology (bottom row).



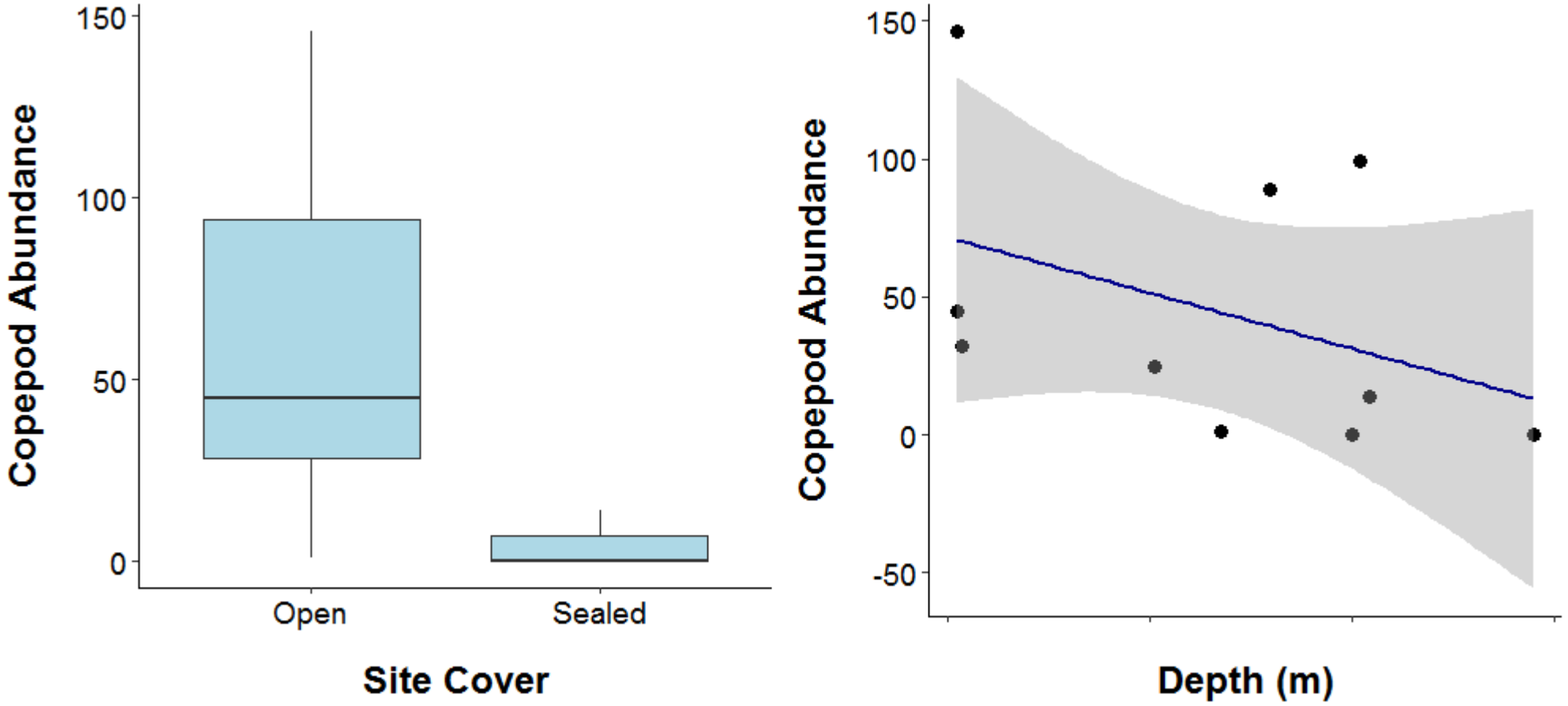
Appendix 3.1 ctd.: Validation plots for copepod biomass linear mixed effects models showing the determinants cover (top row) and superficial deposits presence (bottom row).



Appendix 3.1 ctd.: Validation plots for copepod biomass linear mixed effects models showing the determinants season (top row) and depth (bottom row).



Appendix 3.1 ctd.: Validation plots for copepod biomass linear mixed effects models showing the determinants distance to glacial limit (top row) and permeability of superficial deposits (bottom row).



Appendix 3.2: The effect of site cover (open vs. sealed, left) and depth (m, right) on copepod abundances in wells.

Chapter 4 – Geological controls on stygobite distributions in England and Wales

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Keywords: stygobites, geology, controls, range, generalist, dispersal.

4.1 Abstract

Obligate groundwater fauna (stygobites) are organisms which are uniquely adapted to live in the subsurface, and contribute significantly to the pool of global biodiversity. They are characterised by high levels of endemism and narrow distribution ranges, due to their fragmented habitats and limited scope for dispersal. Although there is a growing body of groundwater research that has investigated the complex controls on stygobite distributions, these controls are still not fully understood and a national-scale survey for the UK has not been conducted. In this study, the distributions of the eight stygobite species in England and Wales were mapped on the outcrops of 11 previously determined geo-habitats, to determine whether they are controlled by geology. Furthermore, the ranges of hydrochemical parameters that different stygobite species occur in were determined. Overall, stygobites were recorded in all geo-habitats, illustrating the wide range of geologies that they can live in. However, stygobite diversity and frequency of occurrence significantly differed between geo-habitats, being highest in the Chalk, and generally substantially higher in karstic than in porous and fractured habitats. Although some species form significant associations with specific geo-habitats, species distributions were generally not related to single habitat outcrops, with most species present in a range of geo-habitats. Furthermore, there were geographical areas of good habitat (mainly of karst nature in formerly glaciated northern England) where species were absent, indicating that dispersal constraints may supersede geology in determining species distributions. These dispersal constraints were also found to limit some species (e.g. *Niphargus kochianus*) considerably more than others (*Niphargus aquilex*), indicating that distribution controls differ between species. All species were found in habitats with a

fairly wide range of hydrochemistries. These ranges were not related to the number of geo-habitats occupied, highlighting the high within-habitat heterogeneity in groundwater. Many stygobite species have existed in England and Wales for millions of years surviving large temperature fluctuations in repeated glacial cycles, which may have made them more tolerant of a wider range of environmental conditions. This may also explain why they are found in very different types of habitat and have larger geographical ranges than most species in mainland Europe.

4.2 Introduction

Globally, geological habitats harbour unique faunal communities, consisting of bacteria, protozoans, meio- and macrofauna, the latter being mostly invertebrates and fish that complete their entire life cycle in the subsurface (stygobites; Gibert et al., 1994). These assemblages contribute significantly to the pool of global biodiversity, often consisting of rare, endemic species found only at single sites (Sket, 2004; Gibert et al., 2009). Stygobites display remarkable adaptations to their low-productivity environment, including low metabolic rates, locomotory rates, extended life cycles and low population densities (Gibert et al., 1994; Coineau, 2000).

The controls on stygobite distributions are complex and vary across a range of temporal and spatial scales (Strayer, 1994; Humphreys, 2008a; Dole-Olivier et al., 2009; Galassi et al., 2009; Robertson et al., 2009). At the macro-scale, operating in deep geological time across hundreds of thousands of years, stygobite distributions are influenced by tectonic plate movements, marine transgressions / regressions (Banarescu,

1992), and major climatic events (Strayer, 1994; Humphreys, 2008a). Large parts of the UK have been affected by two glacial episodes in recent history: The Anglian glaciation between approx. 478,000 – 424,000 ago (Lee et al., 2004) and the Devensian glaciation approx. 71,000 - 10,000 years before present (Walker et al., 1993). At the regional scale, species occurrence may be limited by aquifer type or geology, with species abundance and diversity being generally highest in karstic and porous rocks (Gibert et al., 1994; Hahn & Fuchs, 2009; Malard et al., 2009). At the local scale, groundwater communities are influenced by nutrient and oxygen gradients (Hahn, 2006; Humphreys, 2008a; Bork et al., 2009), micro-scale changes in lithology (Larned, 2012) and ecological factors, such as interspecific competition (Gibert et al., 2009; Knight & Johns, 2015). Because groundwater habitats are highly fragmented, stygobite distributions are thought to be limited by slow dispersal rates across all of these spatial scales (Holsinger, 1993; Strayer, 1994; Lefébure et al., 2007; Dole-Olivier et al., 2009; Larned, 2012).

Geology perhaps provides the highest explanatory power for distribution patterns because its effects operate on all the scales mentioned above (Lefébure et al., 2007; Johns et al., 2015; Weitowitz, in prep.; chapters 2, 3). The fragmented nature of geologies limits faunal dispersal and therefore influences the large-scale species distributions. Geology also determines the amount of physical space available to stygobites and may limit abundance or diversity in geologies with small pore spaces and fractures (Dole Olivier et al., 2009; Hahn & Fuchs, 2009; Weitowitz et al., submitted). Locally, void size and geological permeability influence chemical parameters important to stygobites, including dissolved oxygen (DO), dissolved organic carbon (DOC) and calcium (Datry et al., 2005; Humphreys, 2008a).

The question how and to what extent geology controls stygobite distributions remains an important ecological and biogeographical question in groundwater ecology research. Although groundwater ecosystems have received considerable attention in mainland Europe (e.g. PASCALIS study; Gibert et al., 2009), studies in the UK are relatively scarce (but see Arietti & Edwards, 2006; Robertson et al., 2009; Johns et al., 2015; Maurice et al., 2015). Previous national-scale assessments have reported that the glacial legacy has a major influence on stygobite species distributions, and is the main reason for the low species diversity in the UK (Proudlove et al., 2003; Robertson et al., 2009). Recent studies have focussed on a specific region (Devon & Dorset, Johns et al., 2015) or aquifer (Chalk, Arietti & Edwards, 2006; Maurice et al., 2015), but these studies, together with numerous recent records in the Hypogean Crustacea Recording Scheme (HCRS, 2016), have not been incorporated into a national-scale analysis.

Evaluating how geology affects species distributions has been hindered by the broad resolution of groundwater habitat typologies (karstic, porous, fractured) (Larned, 2012), which may not reflect the true range of habitats occupied by stygobites. There are 421,649 geological units in the UK, which are characterised by high heterogeneity and small-scale variability in grain and fracture size (Bowling et al., 2005; 2007). A more detailed geological habitat (geo-habitat) typology for England and Wales, based on hydrogeological and hydrochemical differences between the rocks, indicated that even higher resolution geo-habitats are likely to differ in their suitability to groundwater ecosystems (Table 4.1; Weitowitz et al., submitted). This study analysed stygobite distribution data dating back to the early 1900's in the context of this untested typology.

The first aim was to determine whether the geo-habitats differ in their ecological importance, by assessing their stygobite diversity and frequency of occurrence.

Previous studies on groundwater communities have found that the constraints imposed by geology are not equally important for all species. For example, some stygobite species are restricted to a single aquifer type (Galassi, et al., 2009) and only few are able to live in fractured habitats (Hahn & Fuchs, 2009). Furthermore, tolerance of copepods to nitrate pollution differs significantly between species (Di Lorenzo et al., 2014). A similar pattern may be true for England and Wales, where the eight stygobite species differ considerably in body size (HCRS, 2016) and assumed dispersal strategies (e.g. *N. aquilex*'s extensive use of hyporheic zones; Johns et al., 2015). Hence, the physical space and void connectivity provided by different geo-habitats are likely to influence each species in a unique way. Assessing geological controls of stygobite distributions can be complicated by the high levels of cryptic speciation in groundwater (Finston et al., 2007; Murphy et al., 2009; Trontelj et al., 2009; Camacho et al., 2011). For example, a previous study determined that three genetically distinct lineages of *N. aquilex* (A1, B and F) and two lineages of *N. fontanus* (A1 and A2) occur in England and Wales (McInerney et al., 2014). The second aim was to assess the distribution pattern of each species (taking into account cryptic lineages of *N. aquilex* and *N. fontanus*) in relation to the outcrop of geo-habitats, to determine whether their distributions are controlled by geology.

In addition to geology, hydrochemical gradients are also thought to control stygobite communities (Datry et al., 2005; Hahn, 2006; Dole-Olivier et al., 2009), because groundwater habitats are mostly dependent on allochthonous energy sources and thus highly oxygen and nutrient limited (Gibert et al., 1994; Gibert & Deharveng, 2002). As

discussed above, geo-habitats in England and Wales greatly differ in hydrochemistry (Weitowitz et al., submitted), and species inhabiting a wide range of habitats (i.e. generalists) may be present in wider hydrochemical gradients than species restricted to few habitats (i.e. specialists). Habitat generalism in surface habitats is linked to increased tolerance of stressed and degraded habitat patches (Verberk et al., 2010; von der Ohe & Goedkopp, 2013). In light of the continuing degradation of the world's aquifers (Mtoni et al., 2013; Templeton et al., 2015), evaluating the hydrochemical gradients of stygobites may be important to inform future studies that investigate the effects of specific pollutants on stygobite species. The third aim was therefore to compare the hydrochemical ranges that different species are found in.

4.3 Methodology

4.3.1 National-scale assessment of stygobite distributions

Data Sources

Stygobite data were collated from a number of sources, including the Hypogean Crustacea Recording Scheme (HCRS, 2016; http://hcrs.freshwaterlife.org/), a UK-wide database of stygobite records with samples dating back to the early 1900's. Further data were taken from a number of published studies with permission from the authors, including 198 samples from a study in the Chalk (see Maurice et al., 2015), 221 samples from a study of 5 different hydro-units in south-western England (see Johns et al., 2015) and 34 samples from a study on limestones and sandstones in England and Wales (chapter three; Weitowitz et al., in prep.). Additional samples collected by the British

Geological Survey (BGS) and the Environment Agency (EA) (unpubl. data) were also incorporated.

The choice of sites in all of the included studies was restricted to previously existing boreholes, wells and springs. Balanced sampling designs were therefore not implemented (but see Johns et al., 2015), complicating the statistical analysis of the data. The projects were carried out by numerous people using different sampling methods. For example, some studies used Cvetkov's (1968) standard net sampling technique (Johns et al., 2015; Maurice et al., 2015), while others collected samples opportunistically during caving expeditions using a variety of methods, such as kick sampling (HCRS, 2016). Furthermore, the older samples from the HCRS lack negative (absence) records. The aims of the paper were therefore addressed using different subsets of data, further explained in the respective methods sections.

The number of sampling sites (Appendix 4.1) and site types (Appendix 4.2) differed greatly between the 11 geo-habitats. Generally, karstic habitats were much better represented than fractured and porous rocks. With 244 sampled sites the Chalk had the best geographical coverage, followed by the Highly Karstic Limestone with 98 sites. The least well sampled geo-habitats were Small-Pore Unconsolidated (9 sites) and Mildly Karstic Limestone (10 sites), while the Large-Pore Unconsolidated category was not sampled at all. The latter sampling gap is most significant, because the Large-Pore Unconsolidated geo-habitat has large pore spaces, high transmissivities and suitable water chemistry (Weitowitz et al., submitted; Jones et al., 2000), and therefore might be expected to provide a good habitat. However, this geo-habitat is restricted to a relatively small area in eastern England, which is far from most previous study areas.

Table 4.1: Summary of geo-habitats from the novel typology for England and Wales (from Weitowitz et al., submitted).

Geo-habitat name	Area (km^2)	Area (%)	log geo-habitat quality score	Range & age of lithologies included
Mildly Karstic Chalk	19,201	12.6	4.21	Cretaceous Chalk
Mildly Karstic Limestone	1,472	0.96	3.1	Permian limestone, dolostone
Moderately Karstic Limestone	4,497	2.94	3.66	Jurassic oolite, corallian limestones
Highly Karstic Limestone	3,151	2.06	3.07	Carboniferous calcarenite, dolomitised limestone
Small-Pore Unconsolidated	13,733	8.99	2.36	Quaternary clay, sand, clay, mud, silt
Large-Pore Unconsolidated	1,490	0.98	2.69	Quaternary gravel and sand
Mixed Sandstone	12,747	8.35	2.98	Permo-Triassic Sherwood Sandstone, Kinnerton Sandstone
Fractured Sandstone	27,817	18.21	2.16	Neoproterozoic Old Red Sandstone, Crackington Formation, Millstone Grit
Igneous Rock	3,621	2.37	2.66	Neoproterozoic andesite, basalt, granite, volcanic
Metamorphic Rock	3,777	2.47	1.68	Neoproterozoic gneiss, mylonite, schist, slate
Mudstones & Siltstones	61,234	40.09	2.46	Neoproterozoic Aylesbeare Mudstone, Mercia Mudstone, coal, ironstone

While the geology and site details were well documented for the BGS records, this was less so for HCRS records. In order to assign each site to the correct geo-habitat, specific site information (e.g. lithology) from the HCRS was consulted and all records from the HCRS database were plotted against the geo-habitat outcrops in ArcGIS 10.1. Additionally, logs of nearby boreholes (http://www.bgs.ac.uk/data/boreholescans/home.html) were assessed, to identify the dominant geological stratum intercepted by each sampling site. Further steps were taken to ensure that each sampling site was assigned correctly: Sites with inaccurate easting / northing coordinates were excluded, because it could not be established to which geo-habitat they belonged. All records from hyporheic zones and shallow sites beneath superficial deposits were excluded, because they do not sample the true bedrock geo-habitats examined in this study.

Geo-habitat suitability for stygobites

Geo-habitat suitability was assessed qualitatively using the entire dataset. ArcGIS was used to map each species' distribution pattern in relation to previously developed geo-habitats (Weitowitz et al., submitted). Species diversity (number of species) and frequency of occurrence (expressed as % of inhabited sites) were calculated for each geo-habitat. To determine whether the habitat quality method applied by Weitowitz et al. (submitted) was predictive of stygobite species diversity or frequency of occurrence, linear regressions were run with habitat quality as predictor, and species diversity and occurrence as response variables. A linear model with confidence interval was also fitted to the data.

Geological controls of stygobite distributions

All statistical analysis was performed in the open source statistical environment R (R Development Core Team, 2016).

For the quantitative analysis, data from the HCRS (lacking negative records) was omitted, resulting in a reduced dataset with more balanced sampling across geo-habitats (Appendix 4.3). Following Johns et al. (2015), a canonical correspondence analysis (CCA) was carried out on the reduced dataset in the R package 'vegan' (Oksanen et al., 2016) to investigate the association of stygobites with geo-habitats. To increase the explanatory power, additional parameters (available for all sampling sites) were fitted as vectors (northing coordinate, depth) and factors (site type) in the CCA. Two separate ordinations were carried out: Species data only and species data with northing, depth and site type as covariates. To assess whether individual variables might explain stygobite occurrence, a forward selection on Bonferroni-corrected p-values ($P < 0.05$) was applied.

Generalised linear models (GLMs) with binomial error structures were run in R to test statistically whether species preferentially associate with certain geo-habitats. Initially geo-habitat was used as a predictor for the presence / absence of all stygobites, then separate GLMs were run for each species in turn. In the binomial GLMs the Chalk was used as the point of comparison because it is the most thoroughly sampled geo-habitat (Maurice et al., 2015), and was assessed to have the highest quality (chapter two, Weitowitz et al., submitted). The distribution of each species was mapped on to the outcrop of the 11 geo-habitats, to determine whether species are distributed throughout their entire outcrop. Also, the number of geo-habitats occupied by each species was calculated.

Stygobite records were plotted in relation to superficial deposits to investigate whether these may influence the species distributions. Using information from the BGS online lexicon (http://www.bgs.ac.uk/lexicon), superficial deposits at the 1:625k scale were grouped into low-permeability and high-permeability categories, depending on their grain size, available pore space size, and permeability. Fine materials with small pore spaces (i.e. clay, sand and silt) have reduced permeability (Jones et al., 2000) and were incorporated into the low-permeability category, while gravel was assigned to the high-permeability category because it consists of larger, more angular material with larger pore spaces (Wentworth, 1922). The distribution ranges for different species were calculated as the longest distance between any two points using the proximity toolset in ArcGIS.

4.3.2 Stygobite habitat chemistry

Data Sources

Hydrochemical and environmental data from a study by Johns et al. (2015) in south-western England were used. Therefore, chemistry data were only available at a regional scale compared to habitat use data, which were available at the national scale. The parameters investigated were dissolved oxygen (DO), dissolved organic carbon (DOC), nitrate, calcium, temperature and the distance to surface water (data from Johns et al., 2015). DO and temperature were measured with a sonde placed near the bottom of boreholes, while the remaining parameters were measured in bailed water samples (Johns et al., 2015). In contrast, stygobites were all collected in net samples integrating

the entire water column. Both abiotic (e.g. DO, Maurice & Bloomfield, 2012; Sorensen et al., 2013) and biotic (e.g. faunal abundance and diversity, Mauclaire & Gibert, 2001; Datry et al., 2005; Hancock & Boulton, 2008) parameters greatly vary with depth. Although stygobites are thought to feed on bacterial biofilm coating the sediments at the bottom of boreholes (Sinton, 1984; Fenwick et al., 2004; Boulton et al., 2003), they have also been recorded from further up in the water column in nets not lowered to the bottom and on CCTV images (Maurice et al., 2015). Therefore, it is uncertain whether the hydrochemistry and fauna are from the same depth of the sampling site (see section 'stygobite water chemistry ranges' in discussion). However, given that the hydrochemical variability with depth and the movement of stygobites along hydrochemical gradients would be similar in all sites, this was regarded as an acceptable drawback.

Water chemistry ranges of stygobites

To assess the water chemistry ranges stygobites were found in, kernel density plots were produced in R. Stygobite occurrence at different concentrations of the hydrochemical parameters was evaluated and their hydrochemical ranges calculated. The relationship between the parameter ranges and the number of geo-habitats could not be analysed statistically, because only 7 data points (each point representing one species) were available for each parameter.

4.4 Results

4.4.1 Geo-habitat suitability for stygobites

Karstic geo-habitats had the highest quality scores (Table 4.1) and generally had higher species diversity and frequency of occurrence than porous and fractured rocks (Figs. 4.1 – 4.3, Table 4.2). All eight known stygobite species in England and Wales were found in Highly Karstic Limestone and Mixed Sandstone, while seven were documented in the Chalk (Table 4.2). The lowest species diversities occurred in the Mildly Karstic Limestone (one species), Igneous Rock and Metamorphic Rock (each two species) (Table 4.2).

The three geo-habitats with the highest stygobite frequency of occurrence were the Chalk (66 %), Igneous Rock (58.3 %) and Highly Karstic Limestone (40 %). Stygobites were also relatively frequent in the Moderately Karstic Limestone (36.8 %) and Small-Pore Unconsolidated rock (33.3 %) (Table 4.2). In Mildly Karstic Limestone (12.5 %), Mudstones & Siltstones (15.4 %) and Mixed Sandstone (16.7 %) stygobites were least common (Table 4.2).

Although habitat quality was predictive of species diversity to some extent, a few geo-habitats had unexpected stygobite diversity. Mildly Karstic Limestone (one species), with a relatively high quality score, harboured much lower diversity than expected. This may be partly due to the low sampling effort in this geo-habitat (Table 4.2), in combination with other factors discussed in the section 'geo-habitat suitability for stygobites'. Although having a low quality score, four species were documented in Mudstones & Siltstones. However, this geo-habitat had the second lowest stygobite occurrence, and *P. cavaticus* only occurred as a single specimen at one site. Overall, both

stygobite species diversity and frequency of occurrence were positively related with the habitat quality score, but these relationships were not significant (Fig. 4.4). In all geo-habitats a number of non-stygobite freshwater taxa (e.g. copepods) as well terrestrial taxa were found (Appendix 4.4).

4.4.2 Geological controls on stygobite distributions

The Canonical Correspondence Analysis indicated a separation of the centroids of the 8 included geo-habitats based on depth, northing, site type and stygobite presence / absence (Fig. 4.5). The all-inclusive ordination was significantly different from the species-only ordination and explained more of the variance in stygobite presence / absence (total of 26 %; Appendix 4.5). All of the included variables (geo-habitat, site type, northing, depth) were significant ($P < 0.05$; Appendix 4.5), with geo-habitat being the most significant predictor ($P < 0.001$). Overall, clear association differences were evident for the species. The species *N. kochianus*, *N. fontanus*, *C. subterraneus*, *P. cavaticus* and *M. leruthi* were associated with the centroids of the Chalk and Highly Karstic Limestone. *N. aquilex* had a stronger association with Mudstones & Siltstones and Small-Pore Unconsolidated rock than other species, and was positively associated with the northing coordinate. *N. glenniei* was positively associated with Igneous Rock.

All model specifications of the binomial GLMs, including model fit, confidence intervals and r^2 are summarised in Appendix 4.7. According to the GLM for all stygobites their occurrence differed between geo-habitats, being significantly more likely to be recorded in the Chalk than most other geo-habitats (Table 4.3; Appendix 4.6). From the

odds ratio (OR) stygobites were less likely to occur in Moderately Karstic Limestone (24 % less), Fractured Sandstone (19 % less), Mixed Sandstone (14 % less), Mildly Karstic Limestone (6 % less) and Mudstones & Siltstones (2 % less), in comparison to the Chalk.

The distribution map for *N. kochianus* shows that 133 of 166 records were from the Chalk, with only few records from limestones and Small-Pore Unconsolidated Rock in southern England (Fig. 4.1a). *N. kochianus* mainly occurred in the Chalk of southern and central England, but was absent from the Chalk outcrop in northern England. Overall, *N. kochianus* was also by far the most commonly recorded stygobite species (166 records). This was supported by the GLM, which indicated that *N. kochianus* was significantly less likely to occur in Moderately Karstic Limestone (17 % less) and Mixed Sandstone (5 % less) than in the Chalk (Table 4.3).

Similar to *N.* kochianus, N*. fontanus* was widespread in the Chalk of central and southern England (55 sites), but it also occurred frequently in the Highly Karstic Limestone in South Wales (52 sites) (Fig. 4.1b). A few records are in Moderately Karstic Limestone and Mudstones & Siltstones. The binomial GLM indicated that *N. fontanus* was significantly less likely to occur in Mixed Sandstone (9 % less) and Fractured Sandstone (8 % less) than in the Chalk (Table 4.3).

The distribution of *N. aquilex* appeared to be much less restricted by geo-habitat. While most records were from the Highly Karstic Limestone (21 sites) and the Chalk (20 sites), it was also widely present in less suitable fractured geo-habitats, including Fractured Sandstone (7 sites) and Mudstones & Siltstones (6 sites) (Fig. 4.1c). In contrast to *N. kochianus* and *N. fontanus*, *N. aquilex* is geographically more widespread from south-western to north-eastern England, and has colonised the relatively isolated

northern Chalk outcrop (Fig. 4.1c). It was the species with most records close to the Devensian glacial limit and the species that occurred in most geo-habitats (9 habitats). Interestingly, the GLM indicated that *N. aquilex* was significantly more likely to occur in the Small-Pore Unconsolidated geo-habitat than in the Chalk (Table 4.3).

N. glenniei was geographically limited to south-western England (Fig. 4.2a), where it was found in a wide range of geo-habitats with most records in Highly Karstic Limestone (15 sites), Igneous Rock (12 sites) and Metamorphic Rock (10 sites). It has not been recorded in the Chalk, because this geo-habitat is not present in its known distribution. The GLM indicated that *N. glenniei* occurrence was not significantly determined by geo-habitat (data not shown).

M. leruthi was mostly recorded in the Chalk (19 sites) and Highly Karstic Limestone (11 sites). Although it was a relatively rare stygobite (43 records), it was geographically widespread with one record in Mildly Karstic Limestone, close to the Devensian glacial limit (Fig. 4.2b). The occurrence of *M. leruthi* was not predicted by geo-habitat in the binomial GLM (data not shown).

P. cavaticus occurred mostly in the Highly Karstic Limestone (40 sites), the Chalk (8 sites) and Moderately Karstic Limestone (3 sites) of southern England and Wales (Fig. 4.2c), indicating a similar preference for karstic habitats than *N.* kochianus, N. *fontanus* and *M. leruthi*. However, these habitat associations were not statistically significant in the GLM (data not shown).

Most records of *C. subterraneus* were from the Chalk in central and southern England (29 sites), and only a few were recorded in Highly Karstic Limestone (4 sites) and

Mixed Sandstone (4 sites). This species mainly occurred in southern England, with one record north of the Devensian glacial limit in Wales (Fig. 4.3a). *C. subterraneus* was the second rarest stygobite species, occurring in only 6.5 % of the sampling sites. The GLM showed that *C. subterraneus* was 16 % less likely to be recorded in Mixed Sandstone compared to the Chalk, indicating a preference for the latter (Table 4.3).

Except for one record in southern England, *A. stammeri* occurred almost exclusively in Highly Karstic Limestone (4 sites) and the Chalk (2 sites) north of the Devensian glacial limit (Fig. 4.3b). *A. stammeri* was also the rarest stygobite species, occurring in only 1.1 % of sampled sites. The low number of records for this species was probably the reason why no significant habitat associations were evident in the GLM (data not shown).

All species had relatively broad distribution ranges, although there are considerable differences between some of them. *N. aquilex* (473.44 km), *M. leruthi* (408.95 km) and *A. stammeri* (394.06 km) had the largest distribution ranges, while *N. glenniei* (190.23 km) and *C. subterraneus* (261.37 km) had a more limited distribution (Table 4.2).

Copepods occurred in all geo-habitats, with the highest prevalence in the Chalk (77 sites), Mudstones & Siltstones (17 sites), Metamorphic Rock (16 sites) and Mixed Sandstone (16 sites). They were also geographically widespread, occurring in south-western, central and northern England and Wales, and in areas north of the Devensian glacial limit (Fig. 4.3c). Copepod presence in the GLM was predicted by geo-habitat and site type. They were significantly more likely to be recorded in Moderately Karstic

Limestone than in the Chalk, and occurred more frequently in wells than in boreholes (Table 4.3).

Different lineages of *N. aquilex* and *N. fontanus* were not restricted to particular geo-habitats. Lineage A1 of *N. aquilex* was geographically widespread and occurred in a range of geo-habitats, including the Chalk outcrop in northern England and Mixed Sandstone, Mudstones & Siltstones and Metamorphic Rock in south-western England (Fig. 4.6a). A similar pattern was evident for lineage A1 of *N. fontanus*, which was found in the Chalk of central and southern England, as well as Fractured Sandstone and Highly Karstic Limestone in southern Wales (Fig. 4.6b).

Geo-habitats in England and Wales are often covered by superficial deposits that greatly differ in permeability. There are relatively extensive east-west areas of highly permeable superficial deposits (Fig. 4.6c), which may act as dispersal routes for species that can inhabit porous habitats.

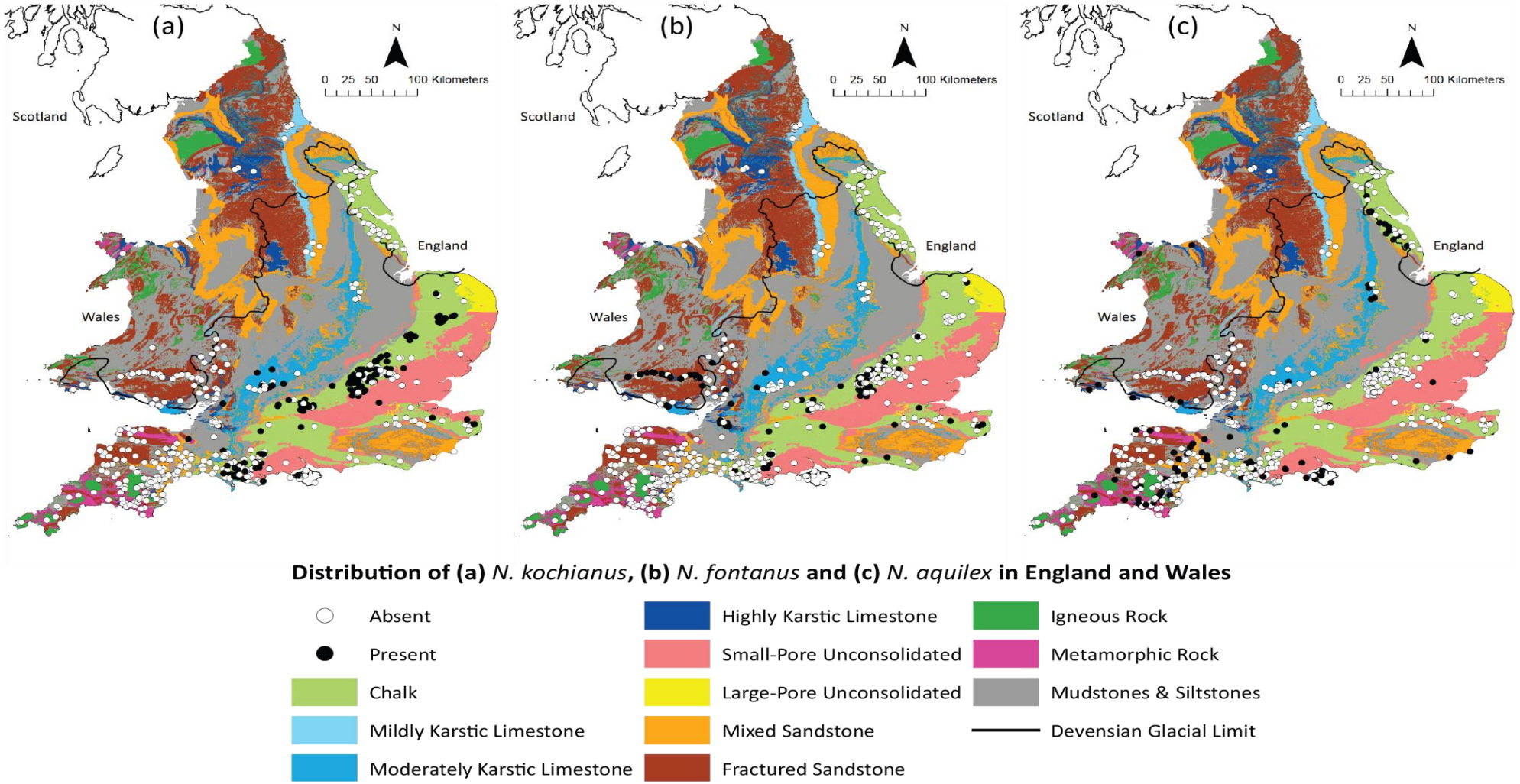

Fig. 4.1: Distribution maps of the species (a) *N. kochianus*, (b) *N. fontanus* and (c) *N. aquilex* in England and Wales.

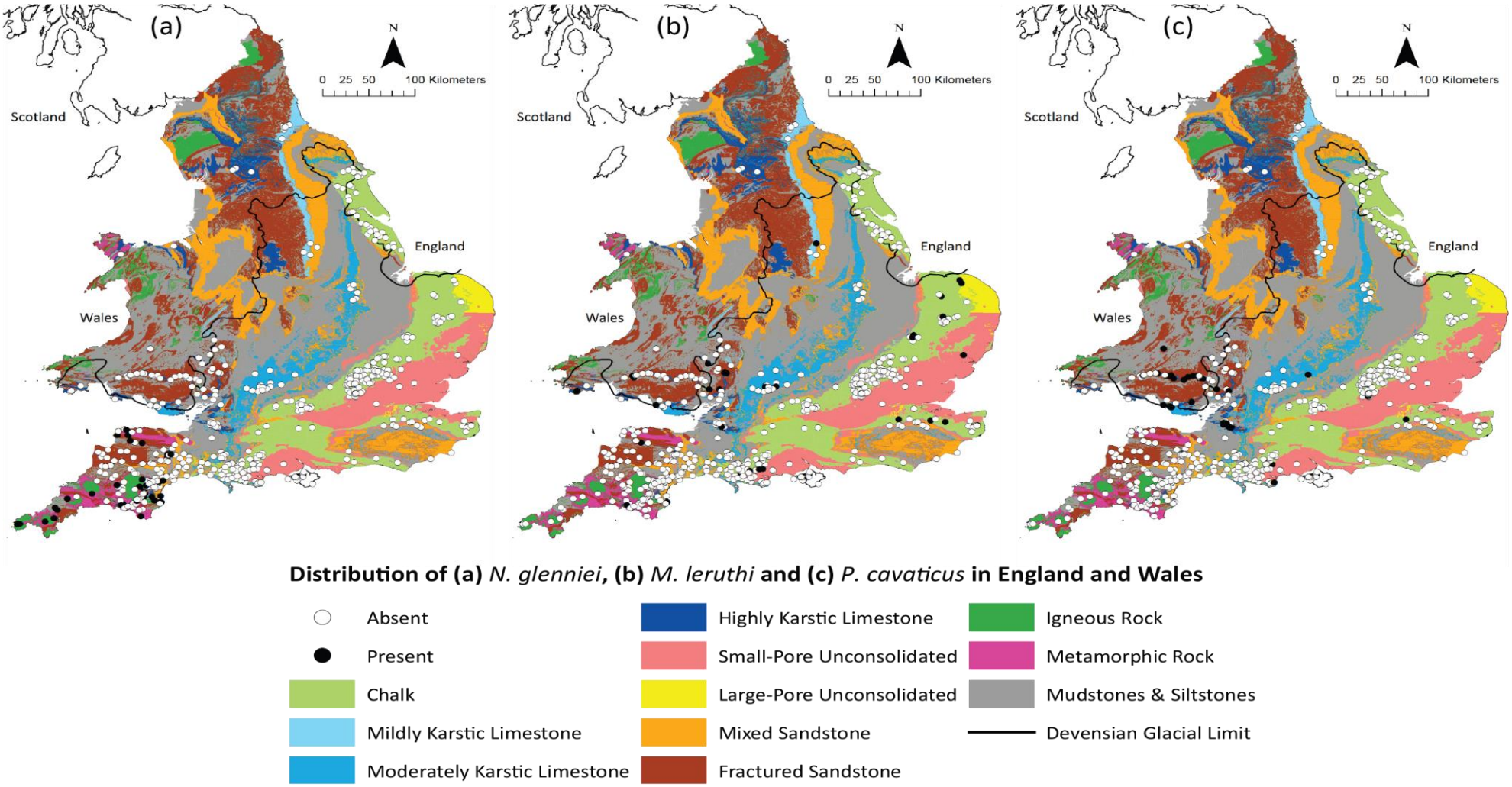

Fig. 4.2: Distribution maps of the species (a) *N. glenniei*, (b) *M. leruthi* and (c) *P. cavaticus* in England and Wales.

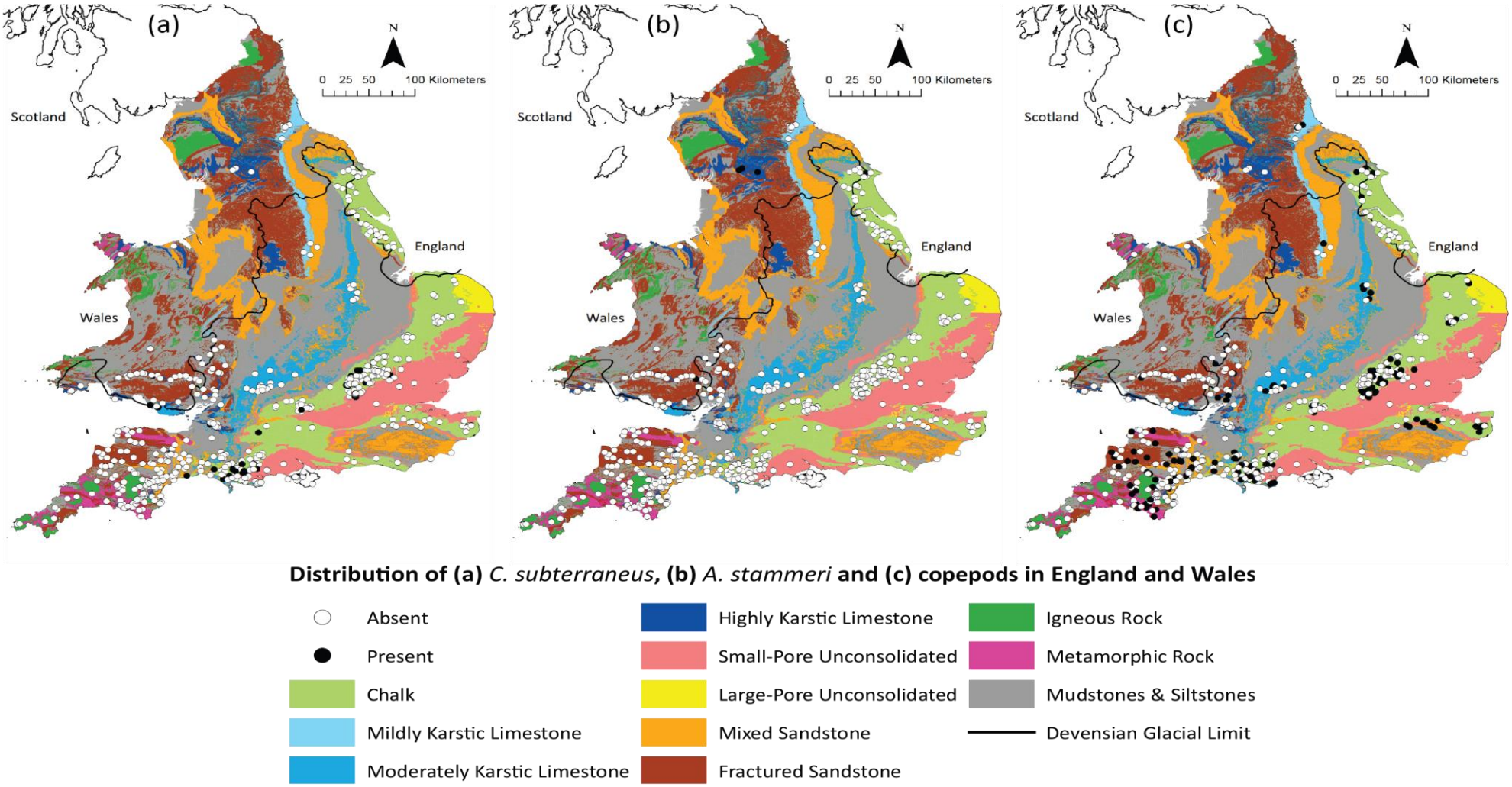

Fig. 4.3: Distribution maps of the species (a) *C. subterraneus*, (b) *A. stammeri* and (c) copepods in England and Wales.

Table 4.2: Summary of the number of sampling sites in geo-habitats, and the stygobite and individual species occurrence across England and Wales. Data were pooled from different sources, including the BGS, EA and Hypogean Crustacea Recording Scheme. The habitat range is given for each species. * The % occurrence of stygobites in geo-habitats was calculated from BGS data only, for which presence / absence data is available (see Appendix 4.3)

Geo-Habitat	Geo-Habitat Score	Total Number of Sampling Sites	Stygobite Records (%)*	*N. kochianus*	*N. fontanus*	*N. aquilex*	*N. glenniei*	*M. leruthi*	*P. cavaticus*	*C. subterraneus*	*A. stammeri*	No. of species
Chalk	4.2	244	171 (66)	136	55	20	0	19	8	29	2	7
Highly Karstic Lst.	3.1	103	98 (40)	3	52	21	15	11	40	4	4	8
Mod. Karstic Lst.	3.7	34	17 (36.8)	8	2	6	0	4	3	1	0	6
Mild. Karstic Lst.	3.1	10	1 (12.5)	0	0	0	0	1	0	0	0	1
Fract. Sst.	2.2	52	16 (24.2)	0	1	7	5	5	0	0	0	4
Mixed Sst.	2.9	56	23 (16.7)	4	2	10	5	1	1	4	1	8
Small-Pore Uncons.	2.4	9	5 (33.3)	1	0	4	0	0	0	1	0	3
Lg-Pore Uncon	2.7	0	NA	0	0	0	0	0	0	0	0	0
Igneous Rock	2.7	23	15 (58.3)	0	0	3	12	0	0	0	0	2
Metam. Rock	1.7	17	13 (34.8)	0	0	3	10	0	0	0	0	2
Mudst. & Siltst.	2.5	33	10 (15.4)	0	0	6	2	2	1	0	0	4
Uncertain	NA	43	43	14	5	25	3	0	1	2	0	6
Total (%)		624	412 (47.4)	166 (26.5)	117 (18.7)	106 (17.4)	52 (8.6)	43 (6.8)	54 (8.6)	42 (6.5)	7 (1.1)	
Habitat Range (km)				293.82	372.21	473.44	190.23	408.95	269.23	261.37	394.06	

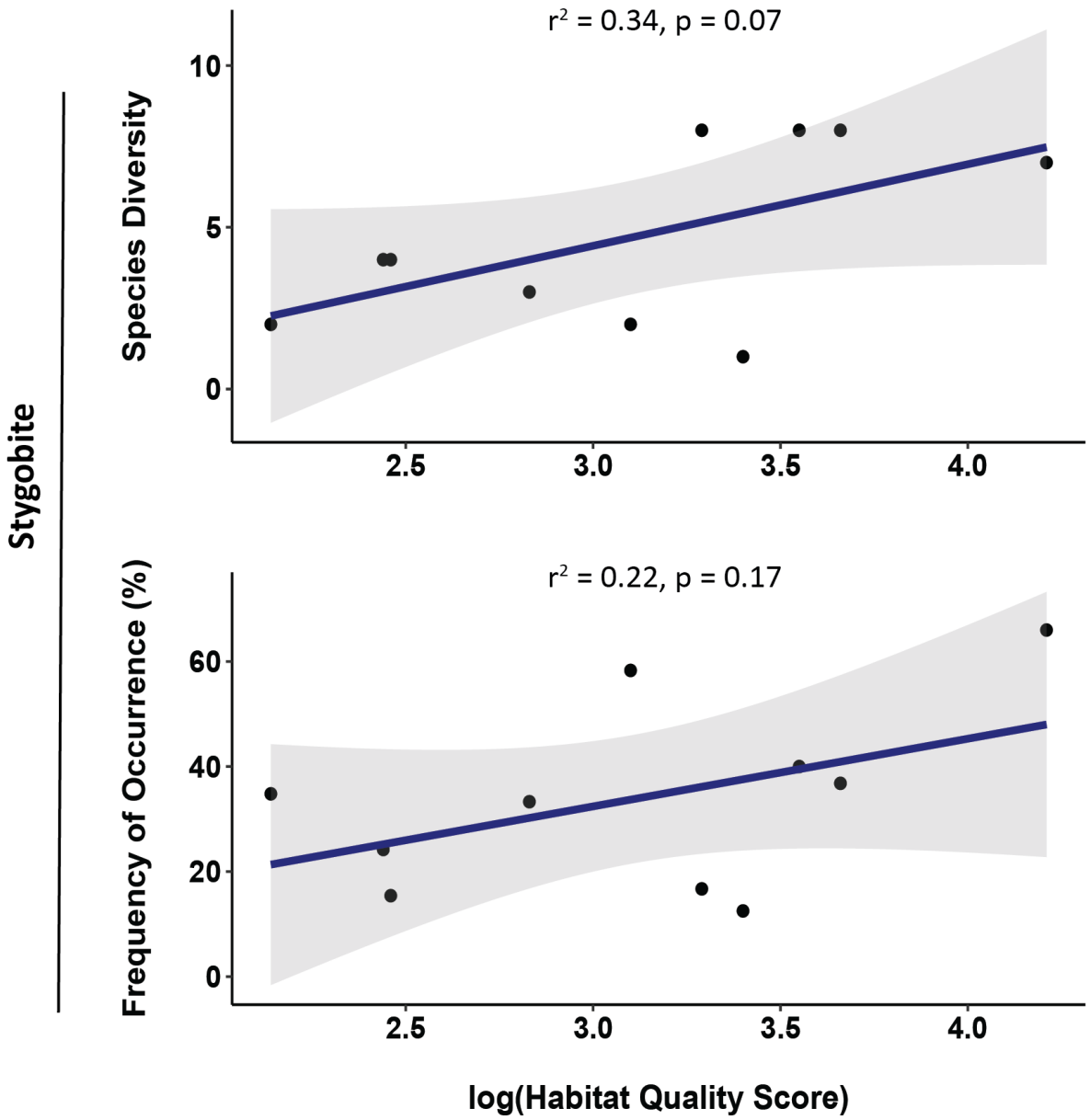

Fig. 4.4: Correlation between the log(habitat quality score) and stygobite (a) diversity and (b) frequency of occurrence. A linear model with shaded confidence interval is applied to the data. The habitat quality score was positively related with both stygobite species diversity and frequency of occurrence, but these relationships were not statistically significant (both $P > 0.05$).

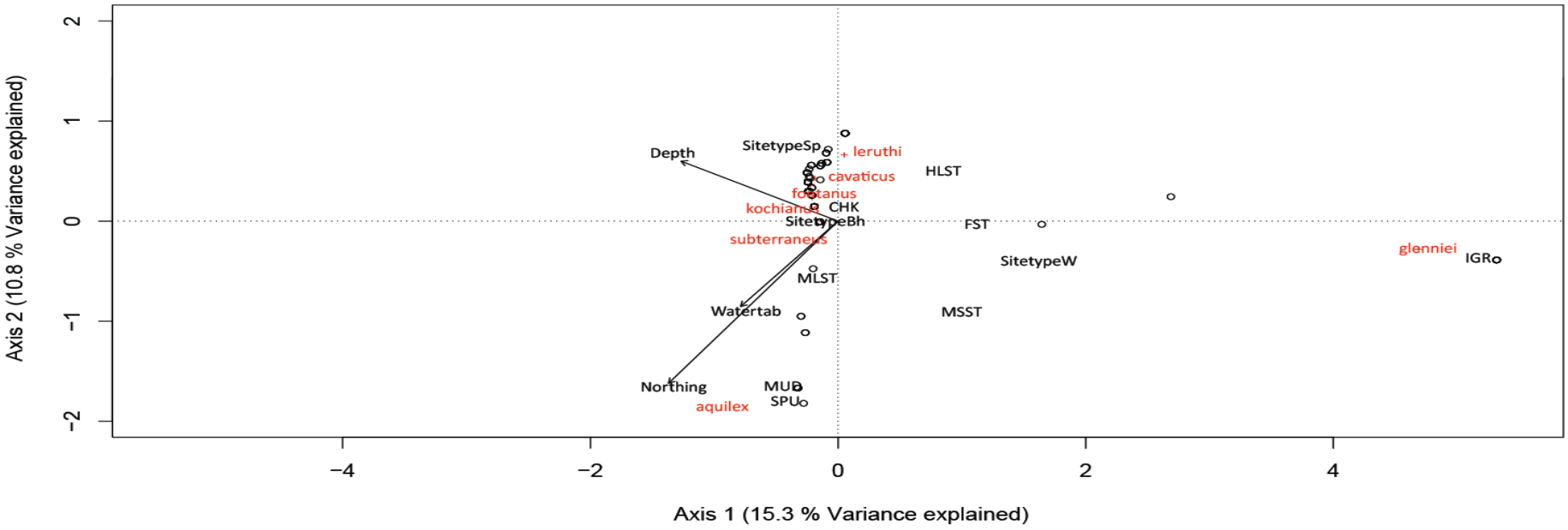

Fig. 4.5: Canonical correspondence analysis (CCA) ordination biplot for stygobite species species with significant environmental variables from sampling sites in England and Wales. CHK = Chalk, HLST = Highly Karstic Limestone, MLST = Moderately Karstic Limestone, FST = Fractured Sandstone, MSST = Mixed Sandstone, SPU = Small-Pore Unconsolidated, MUD = Mudstones & Siltstones, IGR = Igneous Rock, SitetypeSp = Spring, SiteTypeBh = Borehole, SiteTypeW = Well, Watertab = Water Table. Note: Some geo-habitats are missing because environmental data was not complete.

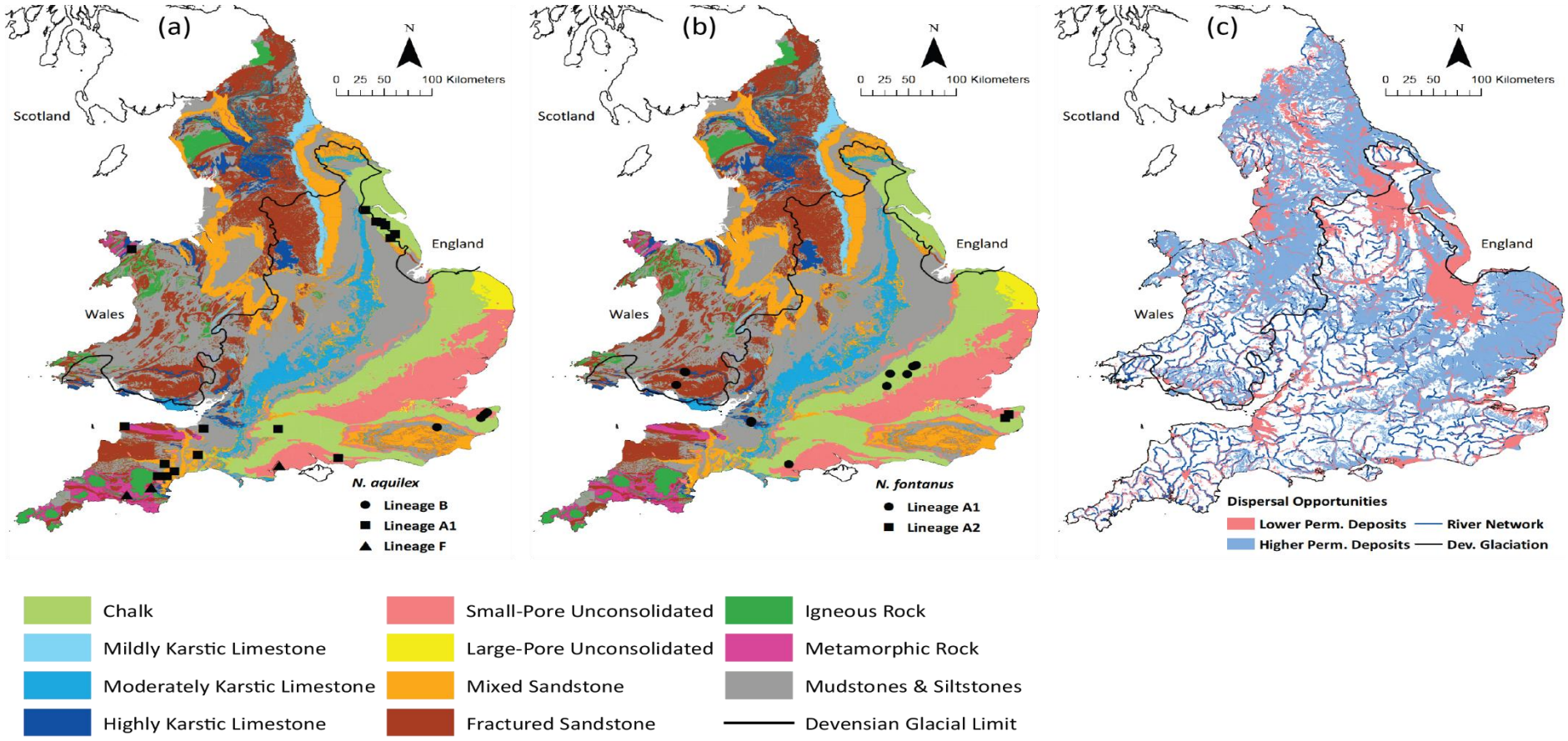

Fig. 4.6: Distribution of (a) different lineages of *N. aquilex* and (b) *N. fontanus* (data from McInerney et al., 2014), and (c) the occurrence of dispersal opportunities (i.e. superficial deposits and rivers) in England and Wales.

Table 4.3: Summary of odds ratios from binomial generalised linear models (GLMs) to predict the presence and absence of all stygobites and individual species from geo-habitat, site type, site depth, depth of water table and the northing coordinate. The simplest possible models were selected based on the Akaike Information Criterion (AIC) and only significant predictors are shown. For the predictor 'geo-habitat' odds ratios are calculated relative to the Chalk, while for site type odds ratios are calculated relative to boreholes. Lst. = Limestone, Sst. = Sandstone, Mudst. = Mudstone, Siltst. = Siltstone, Uncons. = Unconsolidated, bh = borehole

Species	**GLM (AIC)**	**Predictor (cf. Chalk)**	**Estimate**	**Odds ratio**	***P***
All Stygobites	PA ~ Geo-Habitat (AIC = 489.7)	Fractured Sst.	-1.68	0.19	< 0.001 ***
		Mildly Karstic Lst.	-2.86	0.06	< 0.01 **
		Mixed Sst.	-1.96	0.14	< 0.001 ***
		Moderately Karstic Lst.	-1.41	0.24	< 0.01 **
		Mudst. & Siltst.	-3.79	0.02	< 0.001 ***
N. kochianus	PA ~ Geo-Habitat (AIC = 354.3)	Mixed Sst.	-3.09	0.05	< 0.001 ***
		Moderately Karstic Lst.	-1.75	0.17	< 0.01 **
N. fontanus	PA ~ Geo-Habitat (AIC = 262.95)	Fractured Sst.	-2.52	0.08	< 0.05 *
		Mixed Sst.	-2.36	0.09	< 0.05 *
N. aquilex	PA ~ Geo-Habitat (AIC = 217.46)	Small-Pore Uncons.	2.02	7.51	< 0.05 *
C. subterraneus	PA ~ Geo-Habitat (AIC = 200.91)	Mixed Sandstone	-1.82	0.16	< 0.05 *
Copepods	PA ~ Geo-Habitat + Site Type	Moderately Karstic Lst.	0.79	2.2	< 0.05 *
		Site Type (cf. bh)	1.23	3.42	< 0.001 ***

4.4.3 Species habitat chemistry differences

Hydrochemical parameters (e.g. DO, DOC, nitrate and calcium) generally had wide ranges in habitats occupied by stygobites and these ranges often differed considerably between species (Fig. 4.7, Table 4.4). However, habitat generalists (species with more inhabited geo-habitats) generally did not occur in wider chemical ranges than habitat specialists (species with fewer inhabited geo-habitats) (Table 4.4). The DO, DOC, nitrate, calcium and distance to surface water ranges were not related to the number of geo-habitats occupied. For example, *N. aquilex* (9 geo-habitats) and *M. leruthi* (7 geo-habitats), both habitat generalists, had relatively narrow DOC and nitrate ranges, while *N. kochianus* and *C. subterraneus* (both 5 geo-habitats) occurred in considerably higher gradients of DOC and nitrate (Table 4.4).

Temperature was the only abiotic parameter that appeared to be positively related to the number of geo-habitats occupied (Fig. 4.7, Table 4.4). *N. aquilex* (9 geo-habitats), *M. leruthi* (7 geo-habitats) and *N. glenniei* (6 geo-habitats) occurred in the highest temperature ranges, while *P. cavaticus* (5 geo-habitats) and *C. subterraneus* (5 geo-habitats) occurred in the lowest temperature ranges (Table 4.4).

Several species, including *N. kochianus*, *N. fontanus* and N*. aquilex* occurred at sites with very low (< 1 mg/l) and very high DO concentrations (> 10 mg/l; Table 4.4). Similarly, several species, including *N. kochianus* and C*. subterraneus* occurred in sites with very low (< 0.5 mg/l) and very high DOC concentrations (> 290 mg/l; Table 4.4). However, these results need to be treated cautiously as the samples may not be representative of the water chemistry stygobites were actually experiencing in their habitats (see methods).

Table 4.4: Summary of the number and hydrochemistry of geo-habitats containing stygobites in south-western England, showing the number of samples (N), ranges, minima, maxima and mean of parameters. *A. stammeri* was only found at a single site and is not shown.

	N. kochianus **(5 geo-habitats[3])**					***N. fontanus*** **(5 geo-habitats)**					***N. aquilex*** **(9 geo-habitats[1])**					***N. glenniei*** **(6 geo-habitats[2])**				
Parameter	**N**	**Range**	**Min**	**Max**	**Mean**	**N**	**Range**	**Min**	**Max**	**Mean**	**N**	**Range**	**Min**	**Max**	**Mean**	**N**	**Range**	**Min**	**Max**	**Mean**
DO (mg/l)	21	10.21	0.23	10.44	5.38	9	9.34	1.03	10.37	6.14	10	10.29	0.47	10.76	6.44	13	6.86	4.4	11.26	8.44
DOC (mg/l)	24	291.79	0.21	292	36.38	11	34.59	0.21	34.8	7.10	13	12.6	0.2	12.8	3.64	17	14.1	0.2	14.3	2.97
NO3 (mg/l)	22	27.13	2.05	29.18	9.48	11	27.48	1.7	29.18	11.34	10	20.67	1.78	22.45	6.6	13	19.52	1.03	20.54	6.46
Ca (mg/l)	24	73.99	14.18	88.16	48.73	11	88.9	36.47	125.36	57.69	11	81.49	16.88	98.37	46.1	15	41.51	4.85	46.36	28.04
Temp (°C)	37	3.67	10	13.67	11.33	16	3.45	10.05	13.5	11.45	14	6.06	7.85	13.91	10.39	17	5.87	6.63	12.5	9.61
Dist Surf (km)	84	5[H]	0	5	0.7	33	2.86	0	2.86	0.73	16	2.3	0	2.3	0.34	17	0.54	0	0.54	0.17

	M. leruthi **(7 geo-habitats)**					***P. cavaticus*** **(5 geo-habitats)**					***C. subterraneus*** **(5 geo-habitats)**				
DO (mg/l)	4	7.95	1.03	8.98	4.61	5	6.46[L]	0.46	6.92	3.1	9	10.53	0.23	10.76	6.03
DOC (mg/l)	5	3.24	0.2	3.44	1.9	5	18.06	2.54	20.6	10.22	12	178.8	0.2	179	27.43
NO3 (mg/l)	5	15.05	1.74	16.8	8.13	5	10.62	2.96	13.58	7.69	12	25.59	3.59	29.18	10.61
Ca (mg/l)	5	76.95	22.5	99.45	60.32	5	30.57	44.19	74.77	55.87	12	71.79	39.92	111.71	58.65
Temp (°C)	7	4.8	8.7	13.5	10.9	6	3.44	10.20	13.22	11.68	14	3.44	10.47	13.91	11.68
Dist Surf (km)	5	1.88	0	1.88	0.47	6	1.89	0.01	1.9	0.79	28	2.86	0	2.86	0.47

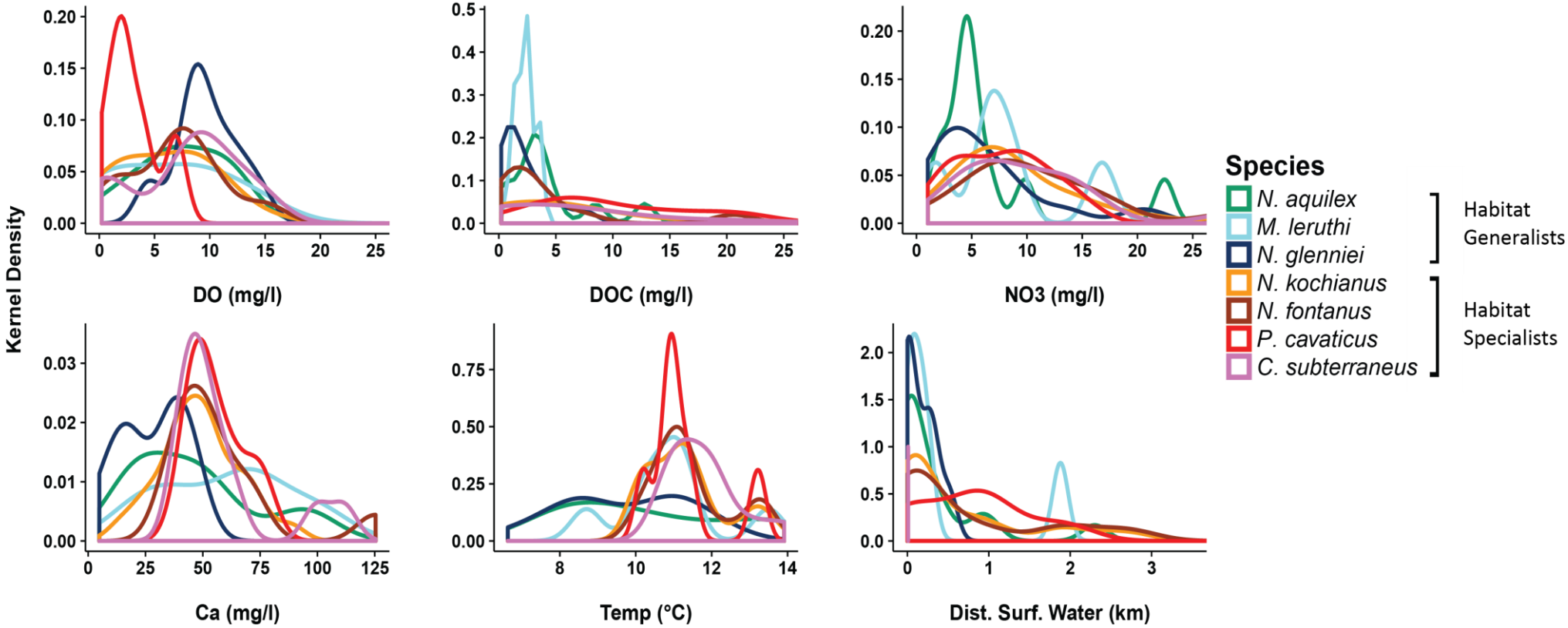

Fig. 4.7: The hydrochemistry of habitats containing stygobites in south-western England. Kernel density values reflect the frequency of occurrence at a particular parameter. Habitat generalists (6 – 9 geo-habitats) are shown in blue colours, habitat specialists (< 6 geo-habitats) in red. *A. stammeri* was only found at a single site and is not shown.

4.5 Discussion

4.5.1 Geo-habitat suitability for stygobites

The Highly Karstic Limestone (8 species), Mixed Sandstone (8 species), the Chalk (7 species) and Moderately Karstic Limestone (6 species) had the highest stygobite species diversities and frequencies of occurrence, which is in accordance with their previously reported high habitat quality scores (chapter 2; Weitowitz et al., submitted). This also supports previous studies that have reported diverse stygobite communities in karstic and porous rocks, many of them being hotspots of biodiversity (Culver & Sket, 2000; Christman et al., 2005; Hahn & Fuchs, 2009; Johns et al., 2015). A particularly good example for this is the Chalk, which harbours abundant communities (Johns et al., 2015; Maurice et al., 2015) and is a vertically extensive habitat with stygobites living at depths of over 70 m below the surface (Sorensen et al., 2013). It was also found to be a good habitat based on hydrogeological and hydrochemical parameters, such as DO and DOC (chapter 2, Weitowitz et al., submitted).

Fractured geo-habitats, which have lower habitat quality scores, had more variable ecological communities. While diversity and frequency of occurrence tended to be lower than in karstic and porous geo-habitats, at least one of these was sometimes reasonably high. For example, while Igneous Rock had low species diversity, stygobites occurred at 58.3 % of sites. Previously, Igneous Rock has been classified as non-aquiferous rock (Cornu et al., 2013), implying that it is a less suitable habitat for stygobites. The high prevalence of *N. glenniei* and *N. aquilex* indicates that this geo-habitat has many good habitat patches, and supports its classification as a moderately suitable habitat in a previous typology (Weitowitz et al., submitted).

Contrastingly, in the Mudstones & Siltstones, stygobite occurrence was very low, but species diversity (four species) was reasonably high. This was surprising, because of the relatively low transmissivities in this geo-habitat (Jones et al., 2000). The relatively high species diversity and reasonable concentrations of DO, DOC and calcium found in this geo-habitat (chapter 2; Weitowitz et al., submitted), indicate that Mudstones & Siltstones sometimes provide suitable habitat patches for groundwater ecosystems. Similar fractured habitats elsewhere harboured depauperate assemblages (Hahn & Fuchs, 2009) and were identified as barriers to dispersal (Johns et al., 2015). The low stygobite frequency of occurrence in the Mudstones & Siltstones supports this, indicating that large parts of this geo-habitat probably have few large fractures, low permeability and poor water chemistry, and most likely slow down dispersal. Stygobites that occur in the Mudstones & Siltstones are most likely restricted to few interspersed patches of more permeable limestones or sandstones (Jones et al., 2000).

This raises the question how individuals in Mudstones & Siltstones reach their habitat patch, in a geo-habitat that is unlikely to be well connected to adjacent geologies. Most likely, stygobites disperse there along more connected corridors near the surface, such as superficial deposits and hyporheic zones (see discussion on individual species later; Ward & Palmer, 1994). Indeed, two of the three stygobite species found in Mudstones & Siltstones (*N. aquilex* and *M. leruthi*) also occur in most habitats generally, suggesting that species that are capable of dispersing into multiple habitats may also colonise Mudstones & Siltstones more easily.

Very unexpectedly, 33.3 % of sites in Small-Pore Unconsolidated rock contained stygobites, although this geo-habitat contains lithologies with pore spaces that were thought to be too small to harbour metazoans (chapter 2; Weitowitz et al., submitted). Similar to the faunal occurrence in Mudstones & Siltstones, stygobites may be restricted to sands, gravels and pebbles that are mixed in sporadically with the clays and silts of this geo-habitat (Jones et al., 2000). The relatively high frequency of occurrence may be related to the preferential construction of boreholes in the most permeable sections of aquifers (Allen et al., 1997), which may be predominantly where more of these coarse-grained materials occur. Recognising the bias of boreholes towards higher yielding sections of aquifers is particularly important for lower quality habitats (e.g. Mudstones & Siltstones, Small-Pore Unconsolidated), because major sections of these geo-habitats are likely to have much lower permeability and depauperate stygobite communities compared to areas with high borehole coverage.

Overall, fractured habitats distinguished in this typology clearly differ in their significance to stygobite communities, supporting the more detailed hydrogeological grouping approach adopted by Weitowitz et al. (chapter 2; submitted) in a response to recent calls for higher-resolution typologies (Larned, 2012; Cornu et al., 2013; Johns et al., 2015). This in turn highlights that it may not be entirely appropriate to utilise typologies with broad groupings (e.g. karstic, fractured, porous; see Hahn & Fuchs, 2009) to analyse stygobite distributions at national scales, because such frameworks may mask geological controls on faunal distributions. For the Mudstones & Siltstones geo-habitat, the geological resolution of the typology was probably too low to explain the occurrence of stygobites. In the case of such highly heterogeneous strata, future regional and local-scale

studies may want to consider more detailed geology and the actual strata intercepted by individual sites, in order to obtain increased explanatory power in their studies.

Despite having a very high quality score, Mildly Karstic Limestone only contained one species and had the lowest stygobite frequency of occurrence. Although the extent of karstification in Mildly Karstic Limestone is lower than in other limestones (Atkinson & Smart, 1981; Worthington & Ford, 2007), it is highly permeable, and expected to provide a high-quality habitat for stygobites (Weitowitz et al., submitted). However, the outcrop of Magnesian Limestone is in northern England and the most likely explanation for its depauperate stygobite community are large-scale extirpations in recent glaciations (Robertson et al., 2009), in combination with the slow dispersal rates in the heterogeneous subterranean environments (Gibert & Deharveng, 2002; Lefébure et al., 2007; Gibert et al., 2009). Furthermore, the surrounding Mudstones & Siltstones are largely poor-quality habitats with low permeability (Fig. 2.7 in chapter 2; Jones et al., 2000), which are unlikely to contain connected dispersal pathways and probably decelerate northward species mobility.

4.5.2 Geological controls on stygobite distributions

There are several significant associations between species and geo-habitats, indicating that at least some species have clear preferences for particular types of habitat. The all-species model indicated that stygobites are significantly more likely to be recorded in the Chalk than most fractured rocks, but also than in most limestones. However, despite this

general preference, the distribution maps and GLMs demonstrate clear differences between species, indicating that geology is not always equally important.

N. kochianus had a significant association with the Chalk and Moderately Karstic Limestone in central and southern England, suggesting a geological control on its distribution. *C. subterraneus* also mainly occurred in the Chalk, although being far less widespread than *N. kochianus*. Interestingly, both of these species were absent from the geo-habitats in southern Wales, although *N. kochianus* has been present in the UK for at least three million years (McInerney et al., 2014) and should have had sufficient time for long-range dispersal. One explanation for this may be that these species have relatively limited dispersal capacities. Both *N. kochianus* (six records) and *C. subterraneus* (eight records) are rarely found in hyporheic zones (HCRS, 2016), indicating they do not use superficial dispersal as frequently as other species. This may be a key factor in constraining their distributions, because the alternative route, long-distance dispersal through bedrock, is very difficult due to the high fragmentation of rock (Galassi et al., 2009; Larned, 2012). This is also supported by the fact that most groundwater habitats in the UK are of poor quality (chapter 2; Weitowitz et al., submitted)

In contrast to *N. kochianus*, *N. fontanus* was not only significantly associated with the Chalk, but also frequently occurred in Highly Karstic Limestone in southern Wales. It was also found at a single site in the Fractured Sandstone, a completely different type of aquifer. Molecular data from McInerney et al. (2014) suggest that individuals from the same genetic lineage (Lineage A1 in Fig. 4.6b) occur on an east-west axis across England and Wales in areas that are geographically distant, and separated by many types of aquifers, including mudstones, sandstones and limestones. Overall, *N. fontanus* is

distributed much further west than both *N. kochianus* and *C. subterraneus*, indicating it may use superficial dispersal routes more routinely. Indeed, *N. fontanus* has been found in hyporheic zones and springs more frequently (36 records; HCRS, 2016).

The distribution of *P. cavaticus* was similar to *N. fontanus*, occurring most frequently in the Chalk and the Highly Karstic Limestone in southern Wales. However, it was not found in Fractured Sandstone adjacent to the Highly Karstic Limestone in southern Wales, suggesting it may be subject to geological controls, such as poorly developed fracture networks or limiting hydrochemistry. The Fractured Sandstone in this area is interspersed with mudstone beds that have few fractures (Jones et al., 2000), and such lithologies have been observed to limit stygobite occurrence elsewhere (Hahn & Fuchs, 2009; Johns et al., 2015). Both *N. fontanus* and *P. cavaticus* are widely distributed throughout the caves of Highly Karstic Limestone in southern Wales and parts of England, although caves have high numbers of endemic species with limited distributions in other areas of the world (Culver et al., 2003; Christman et al., 2005; Niemiller & Zigler, 2013). A possible explanation for the wide distribution of *P. cavaticus* in the caves may be that, similar to *N. fontanus*, it disperses readily in hyporheic zones (HCRS, 2016), which is supported by its large distribution range (> 650 km) across Europe (Eme et al., 2013). The long time periods these species have been present in England (*N. fontanus* approx. 1.5 million years; McInerney et al., 2014) may also contribute to their wide distribution in caves, while the short-range endemics in continental Europe (e.g. Trontelj et al., 2009) may not have had sufficient time to achieve this.

An obvious example of a geological control of *N. kochianus*, *N. fontanus*, *P.* cavaticus, C*. subterraneus* occurs in south-eastern England. While these species are widespread in the Chalk, they appear to be absent from Mixed Sandstone (Lower Greensand in chapter three) that is in partial hydraulic continuity with the Chalk in southern England (Allen et al., 1997; Jones et al., 2000). The Mixed Sandstone in this area is dominated by fine clays and sands with small pore spaces (Jones et al., 2000), which are likely to exclude most metazoans except for smaller-bodied copepods. Pore space size also determined living space and stygobite assemblages in other areas, such as in Cantabria (Spain, Stoch et al., 2004) and the French Jura mountains (Dole-Olivier et al., 2009).

It appears that geological habitat quality controls may be superseded by dispersal constraints, particularly on short geological timescales of 1000s of years. Given the presence of many suitable habitats (Chalk, Moderately Karstic Limestone, Highly Karstic Limestone) in northern England and that stygobites have been present in the UK for up to 20 million years (McInerney et al., 2014), it might be expected that they colonised the north prior to the last glaciation. Their probable absence there indicates that, if this was the case, they did not survive glaciations in subsurface refugia (as has been suggested for some other species, Proudlove et al., 2003; Kristjansson & Svavarsson, 2007), and were extirpated. Species such as *N. kochianus*, *N. fontanus* and *C. subterraneus* appear not to have recolonised the north since the end of the Devensian glaciation, suggesting that dispersal takes longer than 10,000 years even when there is suitable continuous habitat, as in the case of Moderately Karstic Limestone (chapter 3; Weitowitz et al., in prep.). However, the northern outcrops of Mildly Karstic Limestone (5 sites) and Moderately

Karstic Limestone (6 sites) both had a relatively low sampling coverage. It therefore cannot be excluded that other species found in northern England (*A. stammeri*) or in the southern outcrops of these geo-habitats (e.g. *M. leruthi*, *N. kochianus* and *N. fontanus*) may be present there (for further discussion of this see chapter 3).

While geology and dispersal constraints at least partially control some species' distributions, these are clearly less important for others. *N. aquilex* had the widest geographical distribution and occurred in most geo-habitats, which greatly differ in abiotic characteristics (Weitowitz et al., submitted). One of the lineages (lineage A1 in figure 6a) of *N. aquilex* (McInerney et al., 2014) in England clearly does not relate to any particular geo-habitat, indicating that this species has successfully colonised many habitats. One possible explanation is that *N. aquilex* has attained its wide distribution by dispersing through more permeable hyporheic corridors or superficial deposits, which cover large parts of England and Wales (Stanford & Ward, 1993; Ward & Palmer, 1994; Johns et al., 2015). *N. aquilex* is thought to be well adapted to life in superficial habitats (Proudlove et al., 2003; Robertson et al., 2009; Knight & Johns, 2015) and has been recorded in springs and the hyporheos far more often than any other UK species (271 records; HCRS, 2016), clearly supporting this theory. These superficial strata (illustrated in figure 6c) are likely to have had a considerable impact on the distribution of this species, even though the drainage basins (Bridgland & D'Olier, 1995) and overlying deposits (Favis-Mortlock et al., 1997) have changed substantially over the 5-6 million years that *N. aquilex* has existed in the UK (McInerney et al., 2014). The fact that *N. aquilex* is the only stygobite species that has recolonised the northern Chalk outcrop provides further

evidence for its good dispersal capacity; although it cannot be excluded that it survived the glaciations in subsurface refugia.

N. glenniei often occurred in fractured rocks, but was also found in a range of other geo-habitats, such as Highly Karstic Limestone and Mixed Sandstone. Similar to *N. aquilex*, it occurs in a range of geo-habitats with considerably different abiotic characteristics, suggesting its distribution is not geologically controlled. Despite this *N. glenniei* is an endemic, geographically limited to south-western England and has not been able to colonise suitable habitats in central, eastern and northern England. This is somewhat surprising because *N. glenniei* has existed in England for the last 20 million years (McInerney et al., 2014) in an area that was not glaciated. *N. fontanus*, a much larger-bodied species, attained a considerably larger distribution range in a much shorter time period. One possible explanation for this is that *N. glenniei* is less competitive than species in central England (e.g. *N. aquilex* and *N. fontanus*). A recent study found that *N. glenniei* is significantly preyed upon by *N. aquilex*, a considerably larger species (Knight & Johns, 2015). A similar relationship has also been shown for *N. fontanus*, the largest species in the UK, which feeds on the smaller *P. cavaticus* in Welsh cave systems (Chapman, 1993). Stygobite predator-prey relationships may therefore be mainly size-dependent (also see Lustrik et al., 2011), making successful dispersal of a small species to habitats in central England, where larger species dominate, much more difficult. However, to date little is known about the trophic dynamics in groundwater ecosystems and the co-occurrence of species with different body sizes (Maurice et al., 2015) indicates that other factors (e.g. amount of space, species defense and mobility) must also be influential. Additionally, dispersal of species between south-western and central England

may also be obstructed by a belt of Mudstones & Siltstones, acting as a barrier to dispersal (Johns et al., 2015).

Relatively few individuals of *M. leruthi* have been recorded in England and Wales since its recent discovery on the British Isles (Knight & Gledhill, 2010). Due to its small size it may have been overlooked in some samples, and its distribution range may be larger than it currently appears. Nevertheless, *M. leruthi* was found to be geographically widespread and found in a range of geo-habitats, indicating it has good dispersal abilities, tolerates a wide range of conditions and its distribution is probably not controlled by geology. Compared to other species its small body size (1 – 2 mm; HCRS, 2016) might be an advantage when crossing low-permeability rocks and aquifer boundaries. However, numerous records from hyporheic zones (HCRS, 2016) indicate that *M. leruthi* probably also frequently disperses along these strata. Interestingly, although *M. leruthi* is much smaller than *N. glenniei*, its dispersal does not seem to be limited by competition. As discussed for *N. glenniei* above, this clearly indicates that species competitiveness is not solely determined by body size.

In contrast to all other species, the seven records of *A. stammeri* are almost exclusively from north of the Devensian glacial limit, which is remarkable given the pervasive impact recent glaciations appear to have had on most stygobites (see above). Syncarids, such as *A. stammeri*, have particularly low dispersal capacities (Guil & Camacho, 2001), making it unlikely that *A. stammeri* has successfully recolonised northern England when most other species have not. Currently the favoured interpretation is that *A. stammeri* survived the glaciation in sub-surface refugia, when most other species were extirpated in the north (Proudlove et al., 2003; Robertson et al.,

2009). A similar survival of stygobites in subsurface habitats was also documented in other northern European countries, such as Iceland (Svavarsson & Kristjansson, 2006). Currently it appears that *A. stammeri* only survived in a few isolated locations, but the low sampling coverage of northern England may also mean that it is not as rare as currently thought.

The stygobite species distributions illustrate that, although there may be varying levels of geological and dispersal controls, no species is exclusively linked to any particular geo-habitat. This is corroborated by the large geographical distribution ranges of stygobites (ranging from 190.23 km for *N. glenniei* to 473 km for *N. aquilex*), which are larger than those of most species in continental Europe, where linear ranges are often far below 200 km (Lefébure et al., 2006, 2007; Trontelj et al., 2009). A dichotomy between English and continental species was also present for different genetic lineages of the same species. For example, the range of a *N. fontanus* lineage in western France was only 295 km (Trontelj et al., 2009), compared to a 26 % larger range in England and Wales (372 km). Furthermore, the majority of species known from Australia are small-range endemics limited to a single aquifer (Humphreys, 2008b). The multiple glacial-interglacial cycles in the UK (McInerney et al., 2014) appear to have selected for few ancient species with large geographic ranges (cf. high number of endemics in southern / eastern Europe and Australia; Gibert et al., 2009; Malard et al., 2009; Michel et al., 2009), and that can persist in a wide range of habitats and hydrochemical conditions. This may have enabled them to survive the extreme climate changes in recent glaciations. Although little is known about competition in groundwater ecosystems, it is well known that both predation and competition limit the distribution of organisms in freshwater habitats (Polis, 1999;

Jackson et al., 2001; Hänfling et al., 2011). The reduced biodiversity in England and Wales may potentially imply fewer competitive interactions, which may also contribute to the large ranges.

4.5.3 Stygobite water chemistry ranges

Geo-habitats in England and Wales have been shown to provide significantly different hydrogeology and hydrochemistry for groundwater fauna (Weitowitz et al., submitted). All stygobites inhabit multiple geo-habitats and have large distribution ranges, indicating they might be tolerant of a range of abiotic conditions. Indeed, the hydrochemistry from south-western England indicates that they occur over considerable gradients of all hydrochemical parameters. However, the magnitude of hydrochemical ranges was slightly lower than in some surface freshwater habitats (cf. Zhang et al., 2012; Monteith et al., 2014; Halliday et al., 2015), most likely because groundwater is buffered from temporal and seasonal extremes (Gibert et al., 1994). Interestingly, the DOC range over which *N. kochianus* occurred was much higher than the DOC ranges reported in a groundwater baseline study (Shand et al., 2005) and several surface catchments (Boyer et al., 1997; Monteith et al., 2014; Halliday et al., 2015). However, the low median of DOC (4.71 mg/l) for *N. kochianus* suggests that the large range reflects a few individual outliers rather than the general conditions in this aquifer.

The hydrochemical data, particularly DO and DOC, may indicate that most species can occur in low oxygen (DO < 1 mg/l) and nutrient (DOC < 0.2 mg/l) concentrations. Although it is not certain that stygobites were present at the bottom of boreholes where

the hydrochemistry was sampled, stygobites are thought to feed on sediment biofilm there (Boulton et al., 2008). They are therefore likely to spend at least some time being exposed to these low resource conditions. The ability to persist in low-quality habitat patches is probably the result of unique physiological characteristics, including low activity and metabolic rates (Gibert et al., 1994; Coineau, 2000) that stygobites have evolved. A previous study has shown experimentally that stygobites can even survive periods of anoxia (Malard & Hervant, 1999).

It was hypothesized that stygobites living in a wider range of geo-habitats (generalists) would occur across higher hydrochemical gradients than species limited to few geo-habitats (specialists). While this was true for temperature (*N. aquilex* highest, *C. subterraneus* lowest temperature range), most investigated parameters showed only weak or no associations with the number of geo-habitats occupied. This was surprising, because geo-habitats in England and Wales provide significantly different hydrochemical conditions (chapter two; Weitowitz et al., submitted). The main reason for this may be considerable within-habitat variability of hydrochemical parameters, mainly due to lithological heterogeneity across small spatial scales (Larned, 2012). Any given borehole and well intersects a variety of geological features (i.e. fractures or voids), and their morphology significantly affects the prevailing hydrochemical conditions (Sorensen et al., 2013). Similar local-scale lithological variability most likely also influenced copepod biomass and abundance in boreholes and wells (chapter 3; Weitowitz et al., in prep.). However, the absence of relationships, particularly for DO, may also be due to the aforementioned problem with invertebrates and chemistry measurements not originating from the same depth. The concentrations of DO at the bottom of boreholes are likely to

be very low in all of the geo-habitats (Mauclaire & Gibert, 2001; Maurice & Bloomfield, 2012), and this may have more influence on the observed DO ranges than between-habitat differences.

The habitat uses and hydrochemical tolerances of species may have important ecological implications, as habitat generalists may be more adaptable and such species often have a competitive advantage over other species (Kotze & O'Hara, 2003; Julliard et al., 2004; Devictor et al., 2008). Overall, more targeted studies, sampling stygobites and hydrochemistry from the same depths (e.g. in springs or from packered intervals, see Sorensen et al., 2013) are needed to understand how these animals are affected by the abiotic conditions surrounding them.

4.6 Conclusions

This paper shows that while all geo-habitats harbour stygobites, communities differ considerably between them. The most diverse and widespread stygobite communities were found in karstic geo-habitats, such as the Chalk, Moderately Karstic Limestone and Highly Karstic Limestone. Interestingly, even fractured geo-habitats, such as Igneous Rock (high frequency of occurrence) and Mudstones & Siltstones (relatively high species diversity), provided noteworthy habitats. In Mudstones & Siltstones, stygobites are likely to be restricted to infrequent high-quality patches, where higher permeability limestone or sandstone lenses are present, and/or fracturing is denser. The unexpected faunal patterns obtained from the fractured geo-habitats illustrate that broad typologies may not capture sufficient habitat detail to explain stygobite distributions. Higher resolution

geological data need to be considered in regional studies, to fully understand the ecology of highly heterogeneous geo-habitats, such as the Mudstones & Siltstones.

Several species, such as *N. kochianus*, *N. fontanus* and *P. cavaticus* only occurred in a small number of geo-habitats and were absent in directly adjacent strata, such as Small-Pore Unconsolidated rock and Fractured Sandstone, suggesting geological controls on their distributions. However, these controls are sometimes superseded by the low dispersal ability of groundwater species combined with the glacial legacy in the UK. This is illustrated by the fact that some species, such as *N. kochianus*, *P. cavaticus* and *M. leruthi*, are not distributed throughout the entire outcrop of continuous, suitable habitats (e.g. Moderately Karstic Limestone). For the ancient species that may have been present in northern England and Wales in previous interglacial periods, it appears that 10,000 years since the end of the Devensian glaciation have not been sufficient time to (re)colonise northern England. Therefore, the successful dispersal of stygobites across countries is a very slow process, taking millennia.

Lithological dispersal controls are clearly less important for *N. aquilex*, geographically and geologically the most widespread species, and *N. glenniei*, which is geologically widespread yet geographically restricted to south-western England. While all species may, to some extent, disperse along hyporheic corridors and superficial deposits, *N. aquilex* may do so most effectively, achieving its broad distribution and dispersal across extensive areas of poor habitat, such as Mudstones & Siltstones. The differences in species distribution patterns highlight that considering species individually may advance our comprehension of distribution controls in these understudied ecosystems.

The small species pool in England and Wales also consists entirely of widely distributed species with ranges of more than 190 km, which are larger than the ranges of most continental species. Reflecting their wide distributions, all species inhabit multiple geo-habitats, which have been shown to significantly differ in abiotic characteristics (Weitowitz et al., submitted). Stygobites were also found in a wide range of hydrochemical gradients in south-western England, but further work is needed to identify the hydrochemical conditions stygobites experience in boreholes and aquifers. The hydrochemical tolerance ranges of stygobites are important because of the continued anthropogenic disturbance to aquifers, yet have received very little attention to date (Larned, 2012; but see di Lorenzo et al., 2014; Knight & Johns, 2015). Overall, the distinct UK climate legacy may have selected for a limited number of species, which have ancient lineages, broad distributions and tolerate a wide range of habitat conditions.

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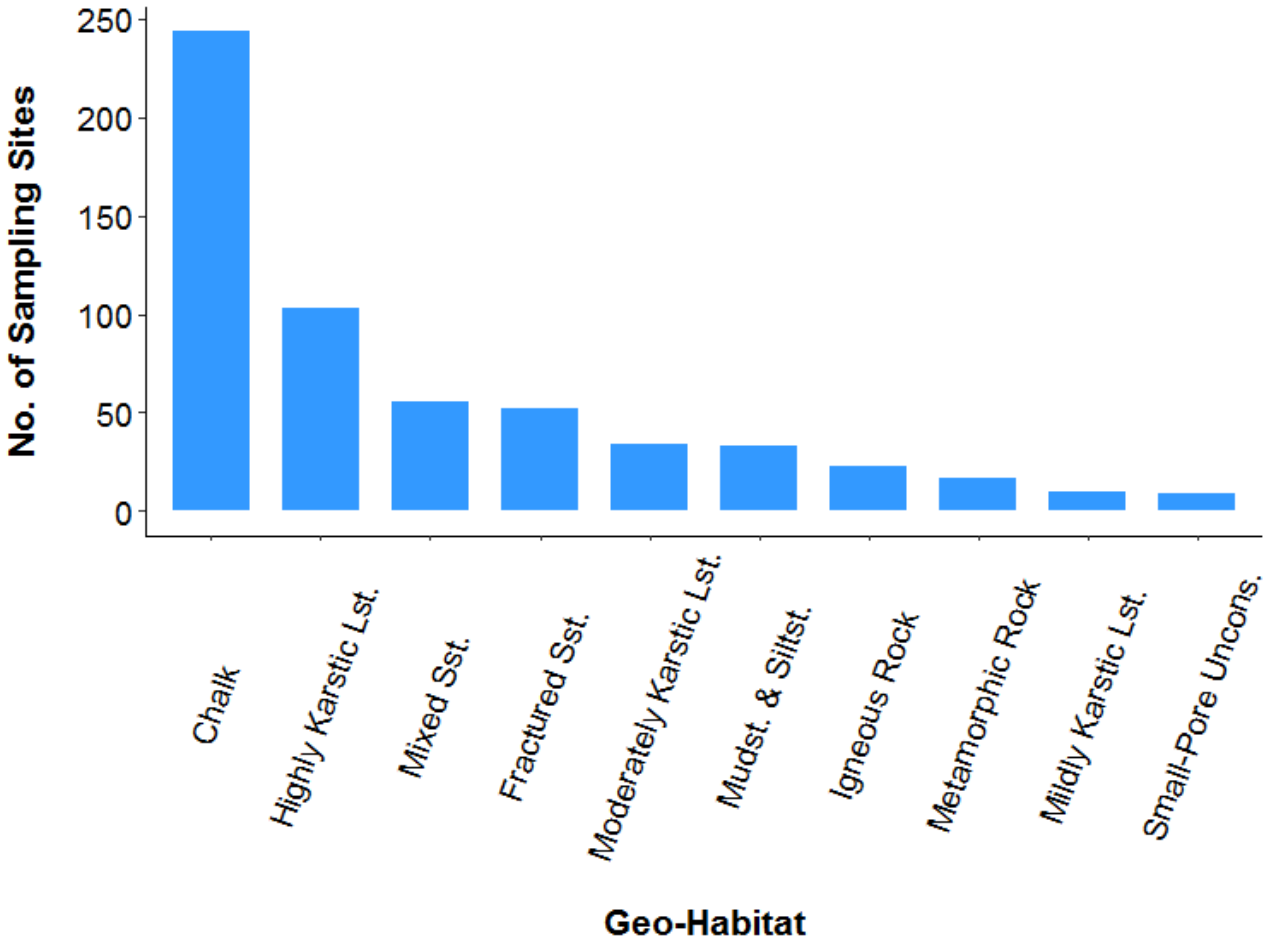
4.9 Appendices



Appendix 4.1: The number of sampling sites in the geo-habitats of England and Wales.

Appendix 4.2: The total number of sampling sites and different site types for each geo-habitat in England and Wales.

Geo-Habitat	Total Sampling Sites	Boreholes	Wells	Spring	Caves	Unknown
Chalk	244	209	29	4	0	2
Highly Karstic Lst.	103	13	1	3	86	0
Mod. Karstic Lst.	34	24	6	0	1	3
Mild. Karstic Lst.	10	10	0	0	0	0
Fract. Sst.	52	29	12	11	0	0
Mixed Sst.	56	20	22	13	1	0
Small-Pore Uncons.	9	8	1	0	0	0
Igneous Rock	23	4	11	6	2	0
Metam. Rock	17	3	8	2	4	0
Mudst. & Siltst.	33	20	6	5	1	1
Uncertain	43	9	18	11	0	5

Appendix 4.3: Summary of BGS stygobite records across geo-habitats in England and Wales for which both positive and negative records are available. These data were used in the statistical analyses of this paper (e.g. CCA and presence / absence GLMs).

Geo-Habitat	**Total Number of Sites**	**Stygobite Records (%)**	***N. kochianus***	***N. fontanus***	***N. aquilex***	***N. glenniei***	***M. leruthi***	***P. cavaticus***	***C. subterraneus***	***A. stammeri***
Chalk	214	141 (66)	112	44	14	0	18	8	28	2
Highly Karstic Lst.	9	4 (44)	0	1	0	1	2	1	0	0
Mod. Karstic Lst.	25	8 (32)	4	0	4	0	4	2	0	0
Mild. Karstic Lst.	10	1 (10)	0	0	0	0	1	0	0	0
Fract. Sst.	49	13 (27)	0	1	4	5	5	0	0	0
Mixed Sst.	42	9 (21)	2	1	2	3	0	0	1	1
Small-Pore Uncon	6	2 (33)	0	0	2	0	0	0	1	0
Igneous Rock	17	9 (53)	0	0	2	7	0	0	0	0
Metam. Rock	5	1 (20)	0	0	0	1	0	0	0	0
Mudst. & Siltst.	24	1 (4)	0	0	1	0	0	0	0	0
Uncertain	2	2 (100)	1	0	1	0	0	0	0	0
Total (%)	403	191 (47)	119 (30)	47 (12)	30 (7)	17 (4)	30 (7)	11 (3)	30 (7)	3 (1)

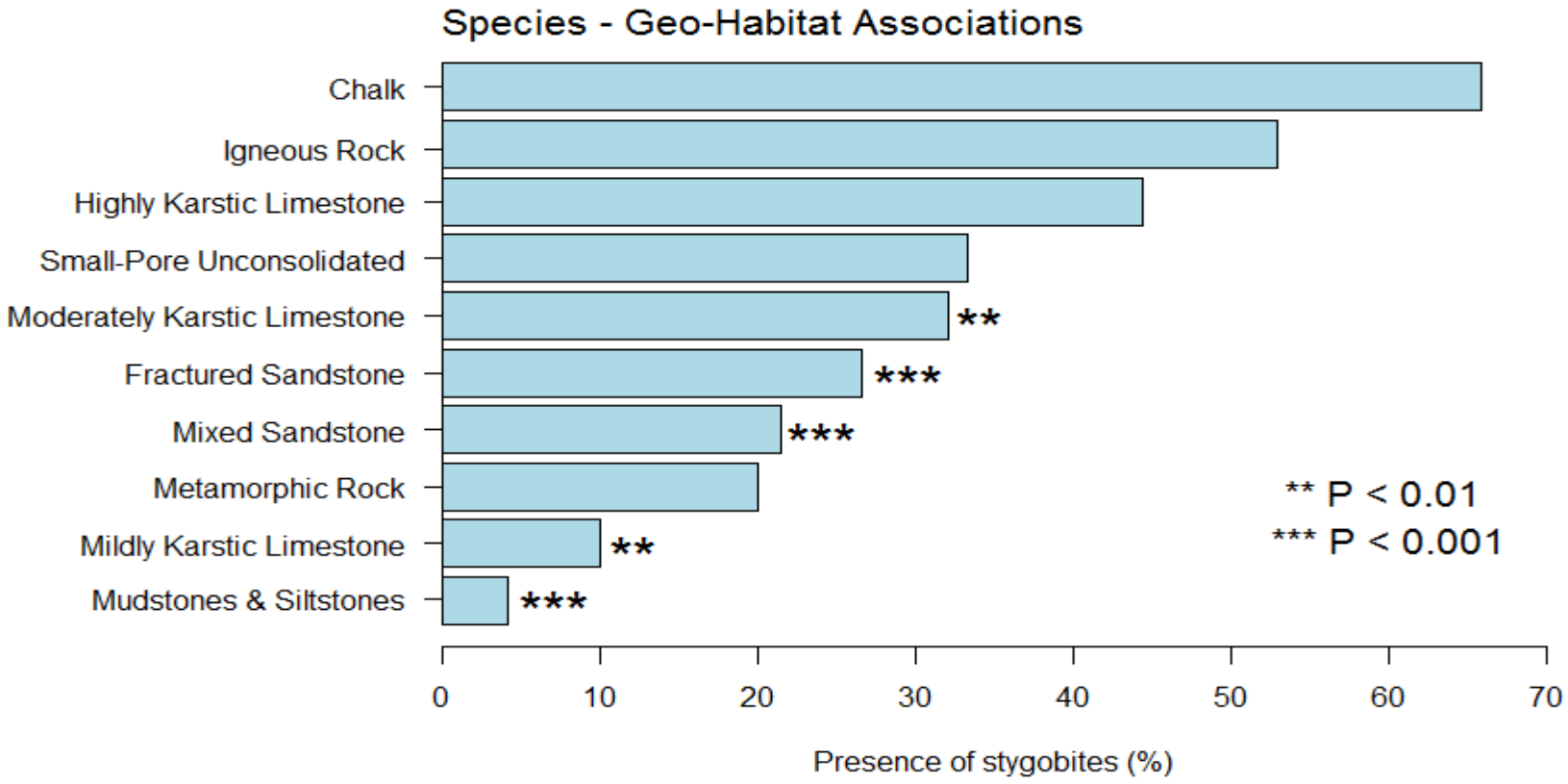
Appendix 4.4: Summary of the occurrence of the most important non-stygobite freshwater and terrestrial taxa in the geo-habitats from BGS data only.

Geo-Habitat	Total No. of Sampling Sites	Non-Stygobite Freshwater Taxa (%)	Copepoda (%)	Oligochaeta (%)	Ostracoda (%)	Acari (%)	Other Taxa (e.g. terrestrial) (%)
Chalk	214	96 (45)	77 (36)	26 (12)	6 (3)	14 (7)	69 (32)
Highly Karstic Lst.	9	6 (67)	5 (56)	2 (22)	2 (22)	0 (NA)	3 (33)
Mod. Karstic Lst.	25	18 (72)	14 (56)	1 (4)	2 (8)	6 (24)	16 (64)
Mild. Karstic Lst	10	5 (50)	3 (30)	3 (30)	0 (NA)	0 (NA)	8 (80)
Fract. Sst.	49	34 (69)	21 (43)	21 (43)	9 (18)	9 (18)	23 (47)
Mixed Sst.	42	30 (71)	21 (50)	18 (43)	9 (21)	15 (36)	19 (45)
Small-Pore Uncon	6	5 (83)	2 (33)	1 (17)	2 (33)	4 (67)	8 (33)
Igneous Rock	17	16 (94)	9 (53)	13 (76)	5 (29)	2 (12)	6 (47)
Metam. Rock	5	4 (80)	4 (80)	4 (80)	1 (20)	0 (NA)	13 (60)
Mudst. & Siltst.	24	13 (54)	10 (42)	11 (46)	7 (29)	3 (13)	16 (67)
Uncertain	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
Total	403	229 (57)	168 (42)	102 (25)	45 (11)	53 (14)	183 (45)

Appendix 4.5: Canonical Correspondence Analysis (CCA) results showing % variance of species data explained by geo-habitat and other environmental variables. The total explained (constrained) variance of the model is given and a comparison (ANOVA) to a species only model is given. The explained variance of species distribution is given for CCA axes 1-4. Significance values based on random permutations of the data are given for each of the fitted vectors / factors.

Overall CCA (F = 5.06, P < 0.001 * cf. species only CCA)**	**Inertia**	**Proportion**
Total	3.45	1
Constrained	1.2	0.35
Unconstrained	2.25	0.65
Contribution of Eigenvalues	**CCA1**	**CCA2**
Eigenvalue	0.53	0.37
Proportion Explained	0.15	0.11
Fitted Vector / Factor	**r^2**	**P**
Northing	0.17	< 0.01 **
Depth	0.07	< 0.05 *
Geo-Habitat	0.34	< 0.001 ***
Site Type	0.12	< 0.01 **

Appendix 4.6: Presence of stygobites (%) as proportion of inhabited sites in geo-habitats of England and Wales. Asterisks (***) indicate a significant difference in presence compared to the Chalk geo-habitat in binomial GLMs.



Appendix 4.7: Verification of significant binomial generalised linear models investigating associations between species and geo-habitats. Shown are overdispersion and r^2 of models and the confidence intervals of geo-habitats cf. the Chalk and wells cf. boreholes.

Binomial GLM	Geo-Habitat	2.5 % CI	97.5 % CI	Binomial GLM	Geo-Habitat	2.5 % CI	97.5 % CI
All Stygobites r^2 = 15.31 Overdisp. = 1.03	Fractured Sst.	-2.4	-1	*N. fontanus* r^2 = 16.16 Overdisp. = 0.8	Fractured Sst.	-5.41	-0.96
	Mildly Karstic Lst.	-5.78	-1.16		Mixed Sst.	-5.25	-0.79
	Mixed Sst.	-2.8	-1.21	*N. aquilex* r^2 = 5.16 Overdisp. = 0.96	Small-Pore Uncons.	-0.02	3.74
	Mod. Karstic Lst.	-2.35	-0.55	*C. subterraneus* r^2 = 15.17 Overdisp. = 0.67	Mixed Sst.	-4.71	-0.23
	Mudst. & Siltst.	-6.69	-2.2	Copepods r^2 = 8.73 Overdisp. = 1.09	Mod. Karstic Lst.	-0.06	1.66
N. kochianus r^2 = 31.22 Overdisp. = 0.72	Mixed Sst.	-4.92	-1.87		Site Type	0.8	1.7
	Mod. Karstic Lst.	-3.01	-0.75				

Chapter 5 – Consumers mediate interactions in a simple groundwater food web

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5.1 Summary

1. Groundwaters are an important resource for drinking water and groundwater communities contribute to the maintenance of this water quality via the breakdown of organic matter, nutrients and contaminants. In groundwater ecosystems, resource supply is scarce and food webs are dominated by few top-level consumers, mainly crustaceans. Many groundwater animals are only found here (stygobites), and thus groundwater communities also make a unique contribution to global biodiversity. These crustaceans clearly feed on groundwater biofilm, but it is uncertain if stygobites can control the abundance and composition of biofilm organisms.

2. We designed two microcosm experiments to explore the feeding effects of three stygobitic crustacean species on groundwater biofilms. Similar to surface waters, we predicted that the crustaceans would influence protozoan and bacterial abundances through grazing, while also changing their community structure. Firstly, we explored how the contrasting feeding behaviours of *Niphargus fontanus* and *Proasellus cavaticus* impacted upon groundwater biofilms over a four-day period. Secondly, we determined the direct and indirect effect of grazer (*Niphargus kochianus*) density on the biofilm. In this second experiment, biofilm responses were measured on nine occasions (bacteria) and six occasions (protozoa) over the course of 32 days.

3. We show that all three species of crustacean stygobites altered the biofilm, significantly increasing protozoan abundances compared to non-grazing controls. In the first experiment, the presence of *Niphargus fontanus and Proasellus cavaticus* significantly increased protozoan abundance. In the second experiment, with *Niphargus*

kochianus, the high grazing density treatment also had significantly higher protozoan abundance and showed more protozoan morphotypes than the non-grazing control and the low grazing treatment. Bacterial densities were not affected by grazing in the first experiment, yet, when bacterial growth was followed over time, significantly different patterns were found between treatments with and without grazers. For example, medium-sized bacteria significantly increased in abundance over time compared to the control when grazer density (*N. kochianus*) was high.

4. Our controlled microcosm experiments are a rare demonstration that macroinvertebrate stygobites can influence and potentially regulate groundwater biofilm assemblages. Therefore, stygobites, through their influence on trophic elements in groundwater food webs, may modulate the denitrification and bioremediation services provided by aquifers and their associated ecosystems. This may contribute to the maintenance of this essential source of clean drinking water.

5.2 Introduction

Groundwaters are an important resource because approximately 2 billion people worldwide depend on it for their drinking water (Morris et al., 2003) and 75% of European drinking water comes from this source (Sampat, 2000). Groundwater communities contribute to the maintenance of this water quality via the breakdown of organic matter, nutrients and contaminants (e.g. Gibert & Deharveng, 2002; Tomlinson & Boulton, 2008), providing a vital ecosystem service (Griebler & Avramov, 2015). Many groundwater animals are only found here (stygobites; Gibert et al., 1994) and thus groundwater communities also make a unique contribution to global biodiversity. Stygobites often have very restricted distributions (Gibert et al., 2009) and persist over long time periods e.g. some *Niphargus* spp. have existed in Europe for tens of millions of years (McInerney et al., 2014).

Food webs in groundwater ecosystems are also unique, because they are truncated and far less complex than their surface counterparts. This is mainly because primary production is minimal in groundwater and resident ecosystems are largely dependent on scarce allochthonous energy to fuel community biomass and production (Gibert et al., 1994; Gibert & Deharveng, 2002). Microbes are the basal component, single-celled organisms (protozoans) and microscopic metazoans are intermediaries and macro-invertebrates (principally crustaceans) or cave fish are top-level consumers (Appendix 5.1). In comparison to related surface water species, stygobites have a reduced metabolism and low growth and reproduction rates, in response to this energy limited environment (Spicer, 1998). They lack eyes and pigmentation and are more resistant to hypoxia and starvation (Hervant et al., 1995; Hervant et al., 1999). Griebler & Avramov

(2015) identified that groundwater food web interactions, especially those between micro- and macro-organisms, are poorly understood (but see Boulton et al., 2008) and represent a major research gap, mainly because few experimental studies with appropriate replication have been conducted. There is also conflicting evidence for top-down control of groundwater food webs by stygobitic crustaceans. Cooney & Simon (2009) found that *Gammarus minus*, a cave amphipod, reduces bacterial activity while others demonstrated that bacteria are more abundant and active when grazed by *G. minus* or *Caecidotea tridentata*, a subterranean isopod (Edler & Dodds, 1996; Kinsey et al., 2007). Other studies found no grazing effects of stygobites, attributing this mainly to low metabolic rates as a result of their energy-limited environments (Foulquier et al., 2010, 2011). Similarly, previous studies have provided contrasting evidence for bottom-up control of groundwater food webs. Foulquier et al. (2010; 2011) found that microbial assemblages were more abundant and active at higher levels of DOC, whereas Weitowitz (in prep., 2016; Appendix 5.2) discovered that higher nutrient concentrations did not result in higher bacterial abundances.

Trophic relationships in surface-water ecosystems have received considerable attention in recent decades (e.g. Sih et al., 1985; Billen & Servais, 1990; Muylaert et al., 2002; Shurin et al., 2012), and clearly both bottom-up and top-down forces are important in structuring ecological communities (McQueen et al., 1989; Menge, 2000). Bottom-up nutrient controls are known to be particularly strong (Lapointe, 1997; Nielsen & Navarrete, 2004; Gaudes et al., 2013), although top-down effects by intermediate consumers such as the amphipod *Gammarus pulex* (Moghadam & Zimmer, 2014) and planktonic cladocerans (Segovia et al., 2014) also remain important in determining abundance and diversity fluxes.

In surface freshwater bodies, crustaceans such as *Gammarus* spp. and *Asellus* spp. are known to hold a central and crucial role in ecosystem functioning both as food for higher trophic levels and in the breakdown of organic material (Graça et al., 1994a, 1994b). They can significantly control biofilm groups such as small metazoans (Rosemond et al., 2001) and algae (Duffy & Hay, 2000; Bruno et al., 2008). While microscopically small crustaceans can feed on considerable amounts of protozoan biomass (e.g. the copepod *Eucyclops serrulatus*, Reiss & Schmid-Araya, 2008), predation on protozoans is not documented for macrofaunal isopod crustaceans such as *Asellus* spp.

Protozoans in surface freshwater systems strongly influence microbial populations in both positive and negative ways. For example, using semi-natural flow cells, Wey et al., (2012) showed that the presence of bacterivorous flagellates increased bacterial area coverage and the number of bacterial taxonomic units (Wey et al., 2012). In two separate experiments on protozoan grazing of biofilm, ciliates and flagellates reduced biofilm thickness by over 60 % and changed bacterial size structure (Huws et al., 2005; Humphreys, 2009). Given the importance and strength of consumer mediated interactions in surface waters, it is likely that such interactions also occur in groundwater ecosystems. Furthermore, grazer density and feeding time are important predictors to consider when investigating the effects of stygobites on groundwater assemblages.

Macrofaunal invertebrate stygobites are the top consumers in most groundwater ecosystems. To elucidate their interactions with protozoa and bacteria in groundwater biofilms we collected three stygobite species (all with body lengths of 8 – 11 mm) and natural biofilms in the field. We selected two amphipod crustaceans *Niphargus kochianus* (Schellenberg, 1932), the most abundant and widespread amphipod species in UK Chalk

aquifers (Maurice et al., 2015), and *Niphargus fontanus* (Bate, 1859), that is found in a wide range of UK groundwater habitats (Johns et al. 2015). The third species was a crustacean isopod, *Proasellus cavaticus* (Leydig, 1871), that occurs mainly in carbonate aquifers (Johns et al 2015). Groundwater amphipods and isopods move and appear to acquire food differently. *N. fontanus* and *N. kochianus* preferentially use their gnathopods to pick up, manipulate and ingest pieces of sediment. In contrast, *P. cavaticus* is a bottom crawler, directly grazing on the sediment surface (pers. obs.).

We then transferred biofilms to microcosms in the laboratory and allowed stygobites to feed on them. We performed two complementary experiments that differed in terms of species used and design:

In a first experiment, we tested the top-down effect of presence or absence of two stygobite species with differing methods of food acquisition (*Niphargus fontanus* and *Proasellus cavaticus*) on the response variables of bacterial and protozoan abundance and diversity. We also tested the bottom-up effect of a nutrient at different concentrations (low, medium, high) on the same response variables. A summary of the methodology (particularly how it was integrated with the grazing element of the experiment), results and interpretation of the nutrient work is provided in Appendix 5.2. We expected that the presence of grazers would significantly reduce bacterial and protozoan abundances and assemblage composition; and that *P. cavaticus* would exert a stronger grazing effect than *N. fontanus* due to the scraping 'lawn mower' feeding strategy that has also been observed in some surface isopods (Naylor, 1955; Jones, 1972). Nutrients were expected to dampen the effects of top-down grazing, fuelling bacterial and protozoan abundances, and community composition.

In a second experiment, we examined the effect of different grazer densities (low and high) of *N. kochianus* on bacterial and protozoan response variables, as well as community respiration. A summary of the methodology and results of the respiration measurements is provided in Appendix 5.3. We expected that higher grazer densities would show stronger effects on response variables and that these effects would become more pronounced as the experiment progressed.

5.3 Methods

The experimental designs and response variables of both experiments are summarised in Appendices 5.2 and 5.4.

5.3.1 Experiment 1: Experimental Setup

Nine *N. fontanus* and nine *P. cavaticus* (Fig. 5.1) were collected on two days in November 2013 and May 2014 from a cave system in Wales (Elm Hole; Lat: 51.81, Long: -3.14) and kept in the dark at 11 ℃ in incubators.

We exposed stone tiles in a borehole (Chalk, Berkshire, UK) to obtain natural groundwater biofilms. Stone tiles of equal size (3.1 x 1.4 cm, www.diy.com) were washed in ultrapure water, placed in mesh nets with a mesh diameter of 500 µm and suspended in the borehole for 3 weeks to allow for biofilm colonisation. On retrieval, tiles were transported to the laboratory in a cool box and stored in the dark at 11 ℃ until use.

In total we ran three treatments (two grazer species and one non-grazing control) in this experiment. Running all treatments with a high replication would have required more than the nine individuals we had for each species. Following previous studies (Nyström & Strand, 1996; Crowder et al., 1997) we therefore used a time block design with 4 blocks, using six individuals of each species at a time. In every block, individuals were randomly assigned to microcosms. We replicated each treatment 6 times per block except for the fourth block, where we ran 9 replicates. This design resulted in 81 microcosms, with each individual being 'reused' 4 times.

Prior to the experiment the crustaceans were starved in filtered water for four days to ensure that all grazers purged their gut. Microcosms were set up in 50 ml glass beakers containing 20 ml of filtered and autoclaved borehole water, and were kept at 11 °C in darkness to mirror groundwater conditions. One tile was placed in each microcosm to provide the grazing substrate for the stygobites and one individual of each of the species was introduced into the respective grazing treatments. Grazers were checked for mortality every 24 hours and two died during the experiment, being replaced with an individual of equal size on discovery.

Each block was terminated after 96 hours when crustaceans were retrieved from the microcosms and the abundance and composition of bacteria and protozoa on the tiles was measured.

5.3.2 Experiment 1: Response Variables

One tile from each microcosm was scraped off in 10 ml of 0.25 µm filtered, autoclaved water using a toothbrush with 10 downstrokes on each side. The samples were then mixed on a magnetic stirrer before further processing.

For Protozoa two 500 µl subsamples of the homogenate were fixed for microscope analysis in a final concentration of 2% glutaraldehyde. Each of these samples was analysed under an Olympus CX 21 microscope using a gridded Sedgwick rafter. Protozoans were counted and measured at x400 magnification. Following Adl et al. (2006) cells were assigned to morphotype categories (Appendix 5.5), including different types of ciliates, flagellates and testate amoebae. A standard taxonomic source for freshwater protozoans was consulted for categorisation (Foissner & Berger, 1996).

For bacterial analysis a 1 ml subsample of the initial homogenate was filtered through a 40 µm filter, and 495 µl of this filtrate were analysed using a C6 flow cytometer (BD Technologies; North Carolina). We conducted prior trials (Appendix 5.6) to set the best possible threshold level to identify bacteria and exclude noise. The primary threshold was set on SSC-H (side scatter) 4000 and a secondary threshold on FSC-H (forward scatter) 8000. A dual threshold applies more stringent conditions before counting a particle, excluding more potential noise (BD Biosciences, 2011, p. 5).

SYTO-9 (Molecular Probes, Life Technologies; Massachusetts) was used to tag bacteria and distinguish them from soil particles (Lebaron et al., 1998; Gasol & Del Giorgio, 2000). After preliminary staining trials (Appendix 5.7), a final SYTO-9 concentration of 5 µM was selected (see also Lebaron et al., 1998). 495 µl of the

prefiltered microcosm homogenate was mixed with 5 µl of SYTO-9 stock solution, giving a total volume of 500 µl for flow cytometric analysis. After addition of the stain the samples were incubated in the dark at room temperature for 15 minutes to allow binding of the stain to the DNA.

Before enumerating bacteria, noise caused by the applied electrical voltage and the running of filtered water was gated out on FSC-H vs. FL-1 (green fluorescence) dot plots (Troussellier et al., 1999). These bacterial gates were kept constant throughout the experiment. Different bacterial size groups were identified according to their clustering along the FL-1 fluorescence axis with large bacterial cells emitting stronger fluorescence signals than small cells, allowing for a discrimination of different bacterial populations (see Troussellier et al., 1999). Each 500 µl sample was run for 1 minute on slow flow to minimise doublet counts.

5.3.3 Experiment 2: Experimental Setup

For experiment 2, 250 individuals of *N. kochianus* (Fig. 5.1) were collected from two boreholes in the Berkshire Chalk aquifer. Collected animals were transported to the laboratory in a cool box and maintained in incubators at 11 °C. In one of the boreholes, two tile sizes (large: 3.1 x 1.4 cm, small: 1.5 x 1.5 cm) were suspended in mesh bags to allow groundwater biofilm colonisation over 5 weeks.

This experiment had three treatments: a control, low grazing density and high grazing density. Nine *N. kochianus* were added to the low grazing treatments and 18 individuals to the high grazing treatments. The densities were based on ecology sampling conducted in the same Chalk aquifer (Weitowitz in prep., 2016). Each treatment had 10

replicates, resulting in 30 microcosms in which tiles were sub-sampled. Over the course of 32 days, protozoans were sampled in 5 random replicates of each treatment on six occasions (days 2, 5, 11, 16, 23, 32; 90 samples total). Bacteria were sampled in all replicates on nine occasions (days 2, 3, 5, 9, 11, 16, 18, 23, 27, 32; 270 samples total).

The microcosms were set up in 250 ml glass beakers, which were filled with 100 ml of filtered and autoclaved water. Two large rectangular tiles for bacterial analysis and six small tiles for protozoan analysis were placed in each of the parafilm-sealed microcosms, before grazers were added to the treatments. Additionally, a single control tile in a mesh bag was suspended in microcosms of all treatments, which the crustaceans were not able to graze on.

5.3.4 Experiment 2: Response Variables

The samples for protozoan analysis were obtained by sacrificing one small tile on each sampling occasion. Protozoan tiles were carefully scrubbed off with a toothbrush in 10 ml of autoclaved water. Samples were then fixed with glutaraldehyde and analysed under a microscope as in experiment 1.

Bacteria were sampled from two large tiles in each microcosm and each time we pooled three 200 µl samples (Appendix 5.8). The bacterial samples were then thoroughly homogenised in Eppendorff tubes, before being processed and analysed in the flow cytometer as in experiment 1.

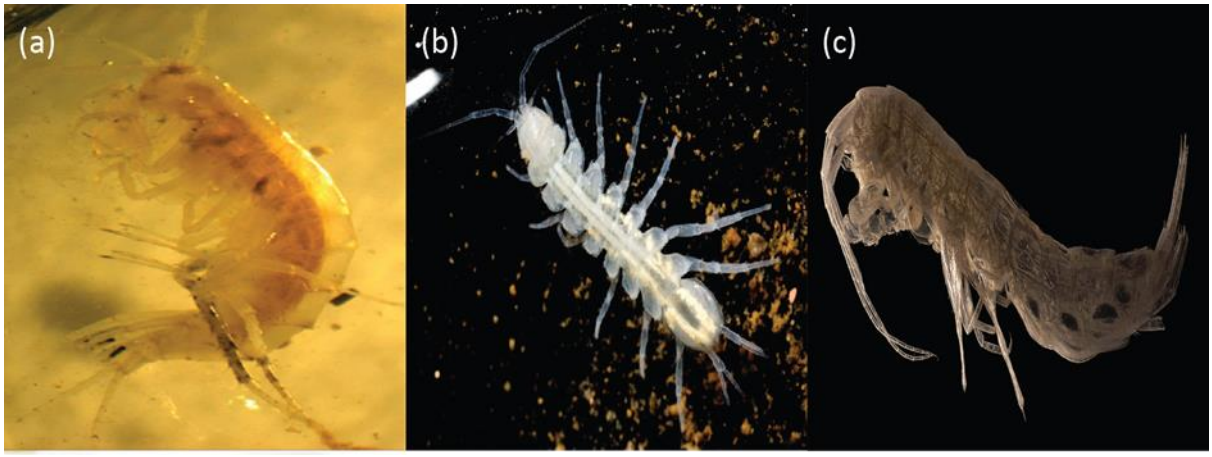

Fig. 5.1: Photos of the stygobite grazer species (a) *Niphargus fontanus* and (b) *Proasellus cavaticus* used in experiment 1 and (c) *N. kochianus* used in experiment 2.

5.3.5 Statistical analysis

Initially all data was checked for normality and homogeneity of variance using the Shapiro-Wilk normality and Levene variance tests. If a response violated parametric assumptions, we used the Box-Cox transformation method of package 'MASS' (Venables & Ripley, 2002) to identify the best power-transformation for the dependent variable.

In both experiments, the effects of grazer present vs. grazer absent was used as a predictor and the response variables were the abundance and composition of bacteria and protozoans on grazing tiles.

We performed a classic analysis of variance (ANOVA) for the data from the first experiment. The predictors were our three treatments (we also fitted 'Block') and the responses were: bacterial abundance, the relative proportion of bacterial size groups, and protozoan abundance and protozoan morphotype diversity (see Appendix 5.4). The two-

way ANOVAs we performed also tested for the interaction of predictors and we refer to predictors and their interactions as 'models' in the ANOVA tables. Due to its extreme non-homogeneity protozoan abundance was analysed using a Friedman's ANOVA with continuity correction (Field et al., 2013).

In the second experiment, repeated-measures ANOVAs using the package 'ez' (Lawrence, 2015) were conducted to test for the effect of different grazing densities, time and their interaction on bacterial and protozoan response variables. In the full ANOVA model repeated measures were fitted as a constant. If the treatment-time interaction was significant, main effects were not investigated even if significant. The effect sizes of each model component were calculated to estimate their relative importance. To evaluate which groups were different we used post-hoc Bonferroni-corrected dependent paired t-tests.

All statistical analysis was performed in the open source statistical environment R (R Development Core Team 2016).

5.4 Results

5.4.1 Experiment 1

The effect of nutrients on bacterial and protozoan responses was non-significant (see Appendix 5.2) and nutrients were therefore not included in the final ANOVA models. The nutrient results are briefly discussed in Appendix 5.2.

The presence of both *N. fontanus* and *P. cavaticus* had a positive effect on protozoan abundances (Fig. 5.2) and this was significant in comparison to the control ($F_{2, 75} = 5.58$, $P < 0.01$; Tukey HSDs, $P < 0.001$; Table 5.1). In *N. fontanus* and *P. cavaticus* microcosms the number of protozoans was double that of the control (Fig. 5.2). Protozoan biomass was higher in both grazing treatments, although this effect was not significant (Appendix 5.9). Block also affected protozoan abundance ($F_{3, 75} = 3.67$, $P = 0.016$; Table 5.1), highlighting the importance of fitting block in the ANOVA.

The top-down control of grazers on bacterial abundance was non-significant ($F_{2, 75} = 1.87$, $P > 0.05$; Table 5.1), although on average bacterial numbers were slightly lower when grazed by either *N. fontanus* or *P. cavaticus* (Appendix 5.9). Grazers did not have any influence on the microbial community composition in terms of changing the relative proportion of small ($F_{2, 75} = 0.8$, $P > 0.05$), medium ($F_{2, 75} = 0.05$, $P > 0.05$) and large bacteria ($F_{2, 75} = 3.4$, $P > 0.05$). Again, block had a significant effect on abundance ($F_{3, 75} = 20.1$, $P < 0.001$; Table 5.1), indicating that bacterial assemblages changed significantly with time and it was important to fit block as a predictor in the ANOVA models

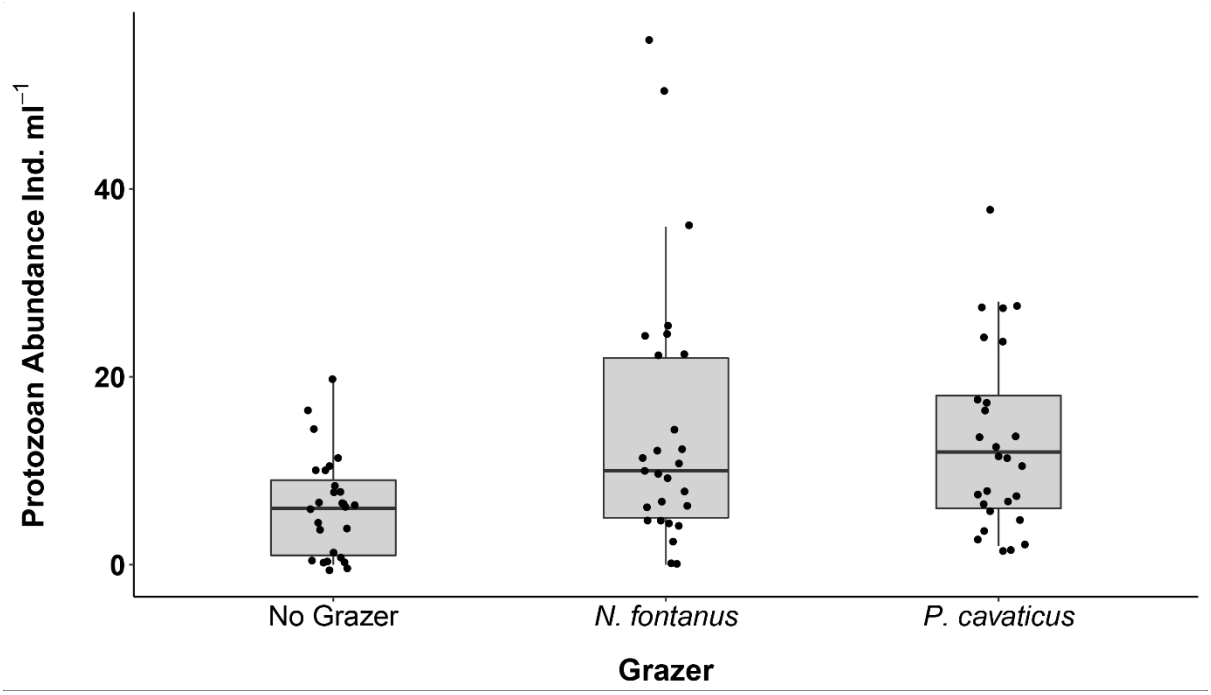

Fig. 5.2: The effect of control, *N. fontanus* and *P. cavaticus* (one grazer in a treatment) on protozoan abundances (ind. ml^{-1}) in feeding microcosms (experiment 1). Boxplots summarise replicates from four different experimental time blocks, with individual data points superimposed to visualise the distribution of the data.

Table 5.1: ANOVA table for experiment 1 in which groundwater crustaceans grazed on biofilm. This experiment had three consumer treatments (control, *Niphargus* present and *Proasellus* present), which had a significant effect on one of the response variables: protozoan abundance.

Model		Protozoan Abundance				Bacterial Abundance			
	df	*SS*	*MS*	*F*	*P*	*SS*	*MS*	*F*	*P*
Consumer	2	1098	548.9	5.58	**< 0.01**	1267	633	2.79	> 0.05
Block	3	1085	361.6	3.67	**0.016**	12880	4293	18.92	**< 0.001**
Residuals	75	7380	98.4			17024	227		
Total	80	9563				31171			

5.4.2 Experiment 2

All bacterial and protozoan measurements are summarised in Appendix 5.10. As in the first experiment, protozoan abundances were significantly affected by the density of the third stygobite *N. kochianus*; and also by time and the interaction of the two predictors ($F_{10, 72}$ = 2, P = 0.048; Table 5.2). Similar to the other two stygobites, the presence of *N. kochianus* resulted in more protozoans in grazing microcosms compared to controls (Fig. 5.3). Averaged for all time points and density treatments, protozoan abundances were twice as high when *Niphargus* was present (Fig. 5.3). Over time, abundances remained at comparably low levels between treatments from day 2 to day 16 (Fig. 5.3). From day 23, protozoans increased in abundance in the high grazing treatment, relative to the control (Fig. 5.3; Appendix 5.11).

The high grazing density treatment also resulted in more protozoan morphotypes than the non-grazing control and the low grazing treatment ($F_{2, 72}$ = 5, P = 0.039; Table 5.2). The number of protozoan morphotypes increased steadily in all treatments over time, with grazed microcosms having slightly higher diversity than the controls (Fig. 5.3; Appendix 5.11).

The density treatments did not significantly affect bacterial abundance ($F_{2, 72}$ = 0.3, P > 0.05), although bacterial abundances were slightly higher in grazed treatments (data not shown). Microbial community structure was significantly affected by the density of *N. kochianus,* by time, and the interaction of the two predictors. In the ANOVA, 'Niphargus Density' was a significant predictor for the proportion of small ($F_{16, 243}$ = 10, P ≤ 0.001), medium ($F_{16, 243}$ = 8, P ≤ 0.001) and large ($F_{16, 243}$ = 6.3, P ≤ 0.001) bacteria (Fig. 5.4, Table 5.2). On day two of the experiment, small bacteria made up a significantly larger

proportion of the total bacterial population in the high grazing density treatment, than in either low grazing or non-grazing conditions (Fig. 5.4; Appendix 5.11). Conversely, the initial relative proportions of medium and large bacteria were significantly higher in the low grazing density and control treatments (Fig. 5.4). Throughout the course of the experiment this trend continuously reversed. Small bacteria dropped under high grazing pressure, while medium and large bacteria increased consistently (Fig. 5.4). In low grazing density and the control, medium and large bacterial size classes showed a slight decline over time. By day 32 the proportion of bacterial size classes was non-significantly different between treatments (Fig. 5.4; Appendix 5.11). Overall, bacterial abundance was also negatively correlated with protozoan abundance, but this relationship was only present under high grazing pressure (Appendix 5.12).

Respiration rates, measured as rate of change in dissolved oxygen, showed no significant differences between treatments after 16 days into the experiment, but were significantly higher under high grazing pressure compared to controls after 32 days of the experiment (Appendix 5.3).

On the mesh tiles excluded from grazing, bacterial abundance ($F_{2,243} = 0.5$, $P > 0.05$), and the proportion of small ($F_{2,243} = 0.1$, $P > 0.05$), medium ($F_{2,243} = 0.02$, $P > 0.05$) and large bacteria ($F_{2,243} = 0.06$, $P > 0.05$) did not differ between treatments (Appendix 5.13).

Table 5.2: ANOVA table for experiment 2 in which the groundwater crustacean *Niphargus kochianus* grazed on biofilm over time. This experiment had three consumer treatments ('control', '*Niphargus* density low' and '*Niphargus* density high'), and time was also fitted as a predictor in the models. Density and Time as well as the models' interaction term are significant in most cases (six response variables).

	Protozoans as response											**Bacteria as response**				
Model	**Protozoan Abundance**						**No. Morphotypes**					**Bacterial Abundance**				
	df	*SS*	*MS*	*F*	*P*	*ES*	*SS*	*MS*	*F*	*P*	*ES*	*SS*	*MS*	*F*	*P*	ES
***Niphargus* Density**	2	931.7	465.9	5.2	**0.037**	0.2	0.5	0.25	5	**0.039**	0.1	146794580	73397290	0.3	> 0.05	0
Time	5	10422.8	2084.6	47.7	**< 0.001**	0.7	8.8	1.76	33	**< 0.001**	0.6	4860136128	972027226	2.3	**0.023**	0.1
Density * Time	10	1440.9	7204.5	2	**0.048**	0.3	1	0.1	0.7	> 0.05	0.1	2412264164	241226417	0.7	> 0.05	0
Error	72	4429.6	61.5				7.02	0.1				52008990000	722347083			
Total	89	17225					17.32					59428184872				

	Bacteria as response															
Model	**% Small Bacteria**						**% Medium Bacteria**					**% Large Bacteria**				
	df	*SS*	*MS*	*F*	*P*	*ES*	*SS*	*MS*	*F*	*P*	*ES*	*SS*	*MS*	*F*	*P*	ES
***Niphargus* Density**	2	82	41	5.1	**0.018**	0.1	1102783	551392	8.3	**< 0.001**	0.1	0.6	0.3	1	> 0.05	0
Time	8	322.5	40.31	6.2	**< 0.001**	0.2	3458568	432321	7.7	**< 0.001**	0.2	29.3	3.7	27	**< 0.001**	0.4
Density * Time	16	885.8	55.36	10	**< 0.001**	0.4	5981887	373868	8	**< 0.001**	0.3	18.49	1.2	6.3	**< 0.001**	0.3
Error	243	1413.8	5.82				11996180	49367				41.1	0.2			
Total	269	2704.1					22539418					89.49				

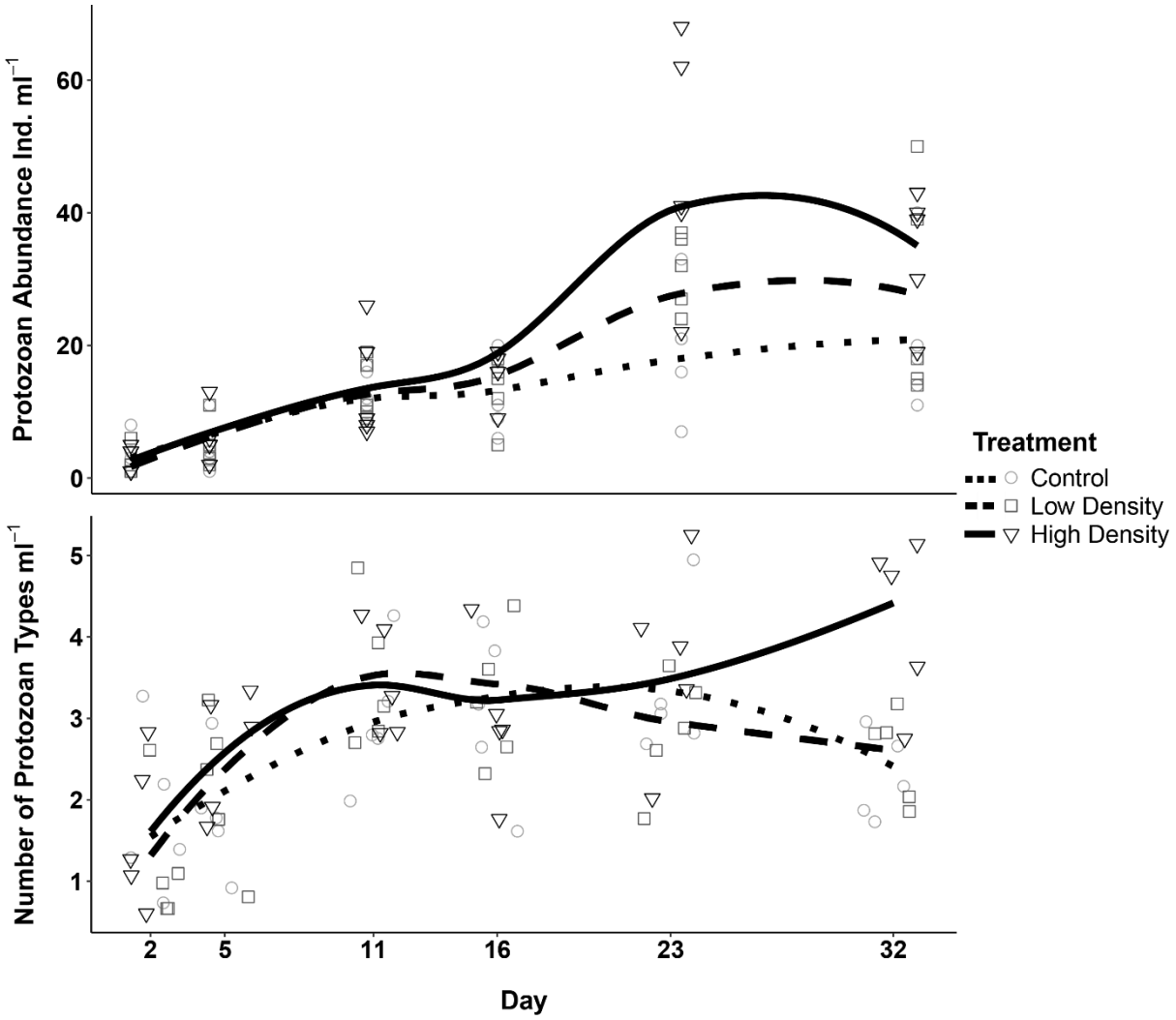

Fig. 5.3**:** The effect of control and different grazing density treatments (low and high) of *N. kochianus* on protozoan abundance (ind. ml^{-1}) and morphotype diversity (no. ml^{-1}) over time in experiment 2. Different grazing treatments are symbolised by dotted (control), dashed (low density) and solid (high density) lines. Protozoan responses were sampled on six occasions (on day 2, 5, 11, 16, 23 and 32).

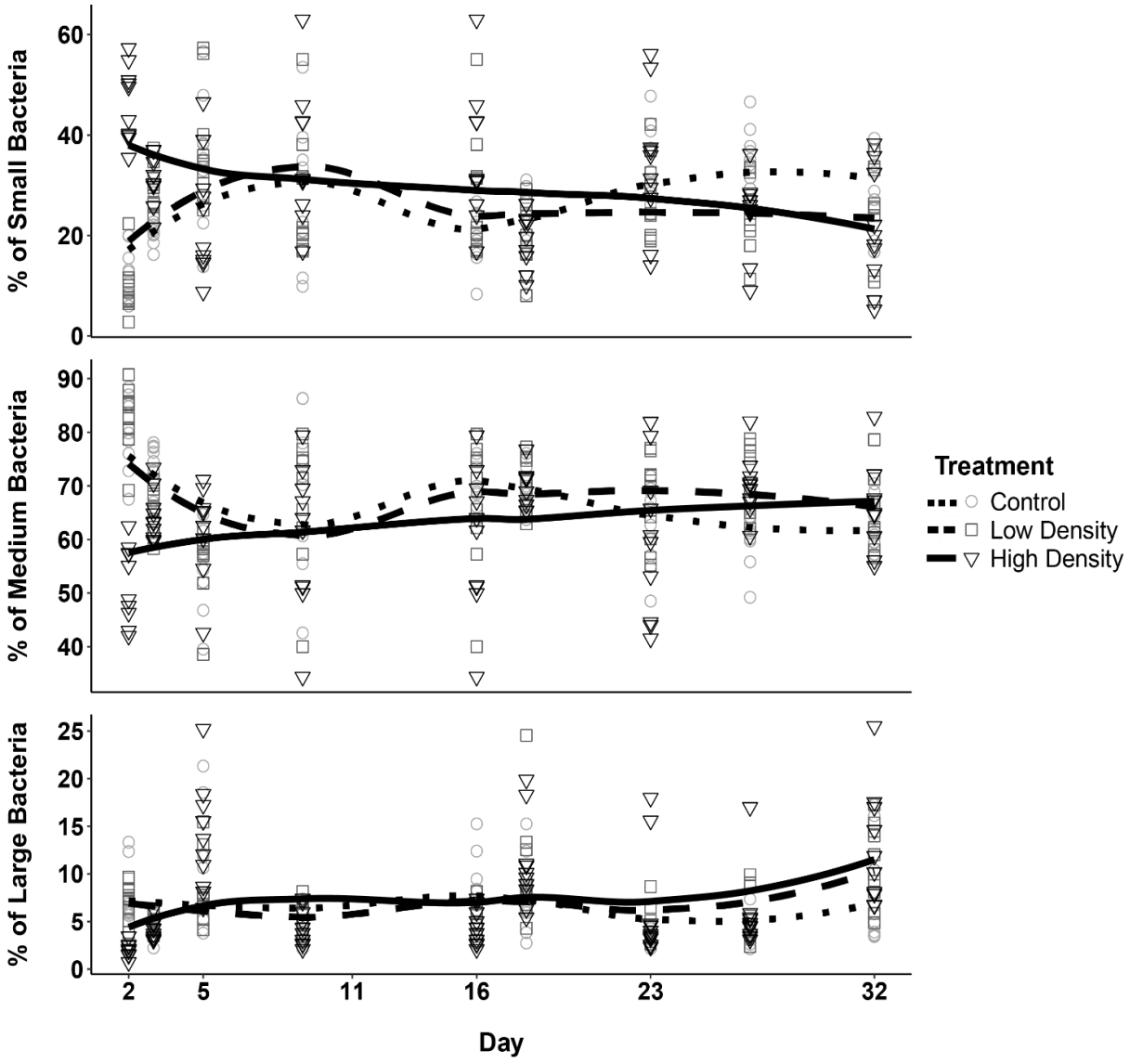

Fig. 5.4**:** The effect of control and different grazing density treatments (low and high) of *N. kochianus* on the relative proportion of small, medium and large bacterial size classes (as % of total bacteria) over time in experiment 2. Different grazing treatments are symbolised by dotted (control), dashed (low density) and solid (high density) lines. Protozoan responses were sampled on nine occasions (day 2, 3, 5, 9, 11, 16, 18, 23, 27, 32).

5.5 Discussion

Our two experiments investigated the impact of three different stygobite species on protozoan and bacterial abundance and species composition. We also examined the response of the biofilm community to differing densities of one stygobite species over time. We were able to show that all three species altered the biofilm, and that the strength and nature of this effect depended on their density and the duration of the experiment. The role of stygobites in groundwater food webs has been intensely debated in recent years (e.g. Boulton et al., 2008). Despite their widespread prevalence and the absence of other top-level consumers, most studies have attributed little importance to obligate groundwater animals in regulating food webs. This has been mainly attributed to the temporal stability of groundwater ecosystems, and the low metabolic rates and perceived low abundances of stygobites (Gibert et al., 1994; Boulton et al., 2003; Wilhelm et al., 2006; Sorensen et al., 2013). However, controlled experiments investigating groundwater food webs are scarce (but see Edler & Dodds, 1996; Cooney & Simon, 2009; Foulquier et al., 2010). Our controlled microcosm experiment is a rare demonstration that macroinvertebrate stygobites can influence and potentially regulate groundwater biofilm assemblages.

5.5.1 Grazing effects on Protozoa

Stygobitic activity increased protozoan abundances in our experimental microcosms. This effect occurred both for single individuals of *N. fontanus* and *P. cavaticus*, and for different densities of *N. kochianus*. In most ecological studies predators tend to reduce their prey abundance (Sih et al., 1985; Mamilov et al., 2000). Studies on microscopically

small crustaceans, such as copepods and cladocerans, have shown that they selectively feed on particular protozoan species (Sanders & Wickham, 1993; Reiss & Schmid-Araya, 2010) and size classes (Stoecker & Capuzzo, 1990; Sommer et al., 2001), demonstrating that these crustaceans can actively hunt for protozoans. In contrast our results indicate that this is not the case for the larger stygobitic crustaceans that we investigated, because protozoan abundances did not decline. Stygobites may graze unselectively on microbial biofilm, and while doing so cause tightly bound biofilm fragments to be dislodged from the substrate (e.g. Gibert et al., 1994). These may be more accessible to bacterivorous protozoans, which thus may reproduce faster and have higher abundances in the presence of stygobites. Such stabilising trends are often observed in grazed microbial populations (Bloem & Baer-Gilissen, 1989; Hahn & Hofle, 2001), but have not previously been demonstrated for protozoans in the groundwater domain.

In contrast to our prediction, *N. fontanus* and *P. cavaticus* did not differ in their effects on biofilm communities (both did not influence bacteria, but increased protozoans), despite having different feeding strategies and mobility patterns. This is perhaps because both feeding behaviours increase the microbial availability to protozoans. *P. cavaticus* may dislodge biofilm segments by browsing over sediment and scraping off bacteria, whereas *N. fontanus*, a very active swimmer, may dislodge biofilm through bioturbation or the handling of sediment pieces.

5.5.2 Grazing effects on bacteria

There was little evidence for the top-down control of stygobites on microbial abundance in our study, although bacteria in biofilm are thought to be one of the main food sources for stygobites (Boulton et al., 2008), and previous work has shown strong positive or negative bacterial responses to invertebrate grazing (Griebler et al., 2002; Cook et al., 2007; Foulquier et al., 2010, 2011). Stygobites have low metabolic rates, adapting them to their resource-limited environment (Gibert et al., 1994). It is therefore possible that the small amount of bacterial biomass removed by grazing is immediately compensated by bacterial growth.

Although stygobites did not affect microbial abundances, we observed time-dependent grazing effects on bacterial community composition. In other aquatic systems protozoan grazing has been shown to affect bacterial community structure (Hahn & Hofle, 1999, 2001), but our work provides the first evidence for an impact of macroinvertebrate stygobites on bacterial community structure. The community structure was affected by grazing from the start of the experiment, but the affected bacterial size classes appeared to respond immediately by increased activity and cell divisions. Such compensation reactions in response to predation have been observed previously, and are the consequence of rapid bacterial generation rates (Hanlon & Anderson, 1979; Traunspurger et al., 1997).

5.5.3 Conclusions

In addition to contributing significantly to global biodiversity (Gibert & Deharveng, 2002), groundwater ecosystems maintain an essential source of clean drinking water. Aquifers and their associated ecosystems additionally provide us with denitrification and bioremediation services (Mattison et al., 2002, 2005; Tomlinson & Boulton, 2008). Our experiments illustrate that stygobites could modulate these processes by controlling activity rates and abundances of protozoa and bacteria through mechanical and trophic interactions. Stygobites may therefore fulfil a crucial role in maintaining food web functionality (e.g. carbon flux) as well as aquifer functionality (e.g. bioremediation). As for species from surface ecosystems, their impact is likely to depend on their abundances in aquifers, which are still not well known (Maurice & Bloomfield, 2012; Sorensen et al., 2013). Stygobites may also provide direct food sources for microbes and protozoa, by secreting faeces or producing pellets of fine interstitial materials (Boulton et al., 2008) and we often observed that stygobites bioturbated and generated pellets in our experiments.

Ecosystem stability is becoming increasingly important in the face of environmental pollution and global climate change. Groundwater communities, and particularly stygobites, are adapted to the constant temperature and low-nutrient conditions in groundwater. A change in groundwater temperatures or nutrient levels may therefore lead to the disappearance of whole functional groups of organisms in these simple systems, leading to groundwater ecosystem destabilisation (Avramov et al. 2013). Further micro- and mesocosm experiments are needed to link groundwater food webs to

ecosystem services, and to underpin an informed approach to the conservation of these ecosystems.

5.6 Acknowledgments

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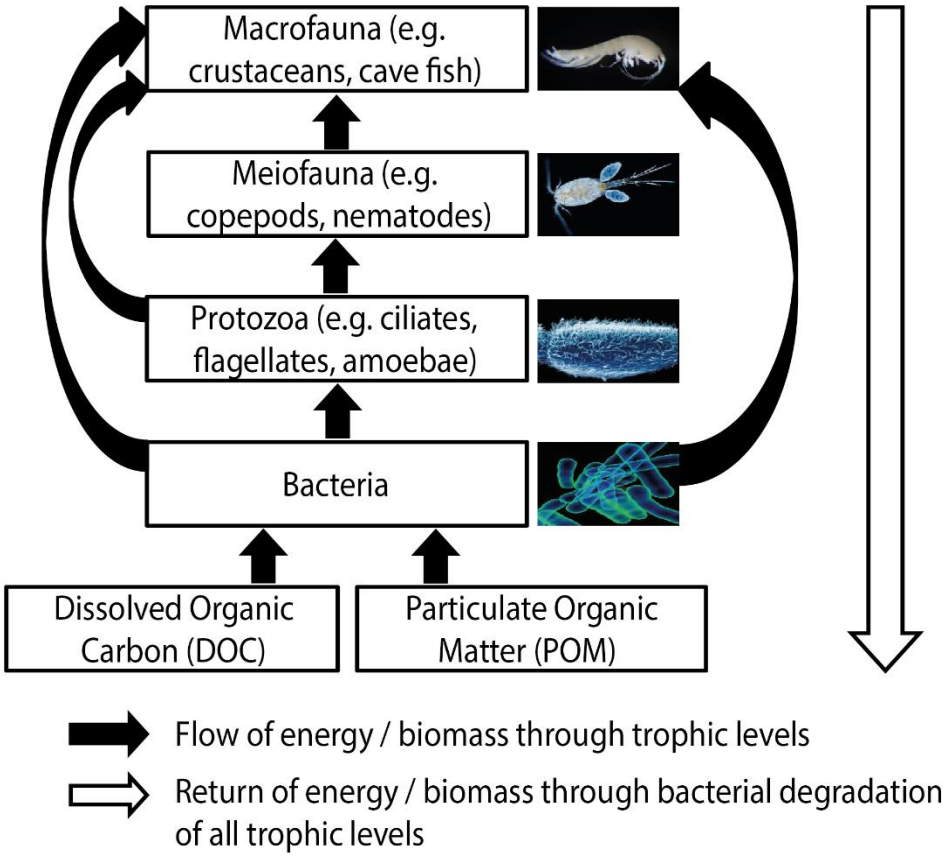
5.8 Appendices



Appendix 5.1: Flow of energy through a typical groundwater food web.

Appendix 5.2: Methodology, results, interpretation and references of the bottom-up nutrient component that was not included in the published version of the experimental study.

Introduction:

In addition to investigating the top-down grazing effect of stygobites, we also wanted to elucidate the role of bottom-up nutrients effects on groundwater biofilm, and to investigate a possible interaction between bottom-up and top-down forces. In surface aquatic ecosystems bottom-up and top-down controls have been found to be equally important in structuring ecological communities (McQueen et al., 1989). In many previous studies, nutrient increases generally had positive effects on microbial biomass and respiration (Findlay et al., 1993; Hall & Meyer, 1998; Craft et al., 2002; Paerl, 2008). Nitrogen and phosphorus addition also resulted in higher abundances and biomass of polychaetes and crustaceans in benthic communities (Posey et al., 1995). Only few studies have investigated bottom-up control in groundwater communities and these found either positive effects of nutrient addition on bacterial abundance and biomass (Cooney & Simon, 2009; Foulquier et al., 2010) or no bacterial response (Edler & Dodds, 1996). All of the groundwater studies used carbon, whereas a form of nitrogen (N) has not been tested as a food source.

Methods:

Each experimental beaker contained 20 ml of ultrapure water with a final nutrient concentration of 1 mg/l (low), 23 mg/l (medium) and 46 mg/l (high) ammonium nitrate (Sigma-Aldrich, >98 %). Ammonium nitrate (NH_4NO_3) is a nutrient that is frequently used to investigate the effects of nutrient loading or eutrophication on ecological communities.

These nutrient concentrations were chosen to reflect the minimum, median and maximum nitrate concentrations measured in a Chalk aquifer (Shand et al., 2003). After nutrient addition each experimental beaker was thoroughly mixed to ensure the even distribution of nutrients in the liquid. Single individuals of either *N. fontanus* and *P. cavaticus* were added to the grazing treatments.

Due to the limited number of grazers available, only 2 replicates of each nutrient * grazer treatment were run at the same time and individuals were 'recycled' in successive experimental blocks (Appendix 5.2.1). Each stygobite was only subjected to each nutrient concentration once (e.g. individual *N. fontanus* 1 was placed in low nutrients in block 1, medium nutrients in block 2, etc. This ensured that individual differences were not a source of variation between nutrient concentrations. Overall, nine replicates over four experimental blocks were run for each grazer * nutrient combination, resulting in a total of 81 microcosms.

Appendix 5.2.1: Factorial design of experiments 1 and 2 showing the number of replicates for each combination of grazer presence and nutrient concentration (experiment 1) or grazing density (experiment 2). Because nutrients had no significant effect on either bacteria or protozoa, they were not included in the paper's ANOVA models.

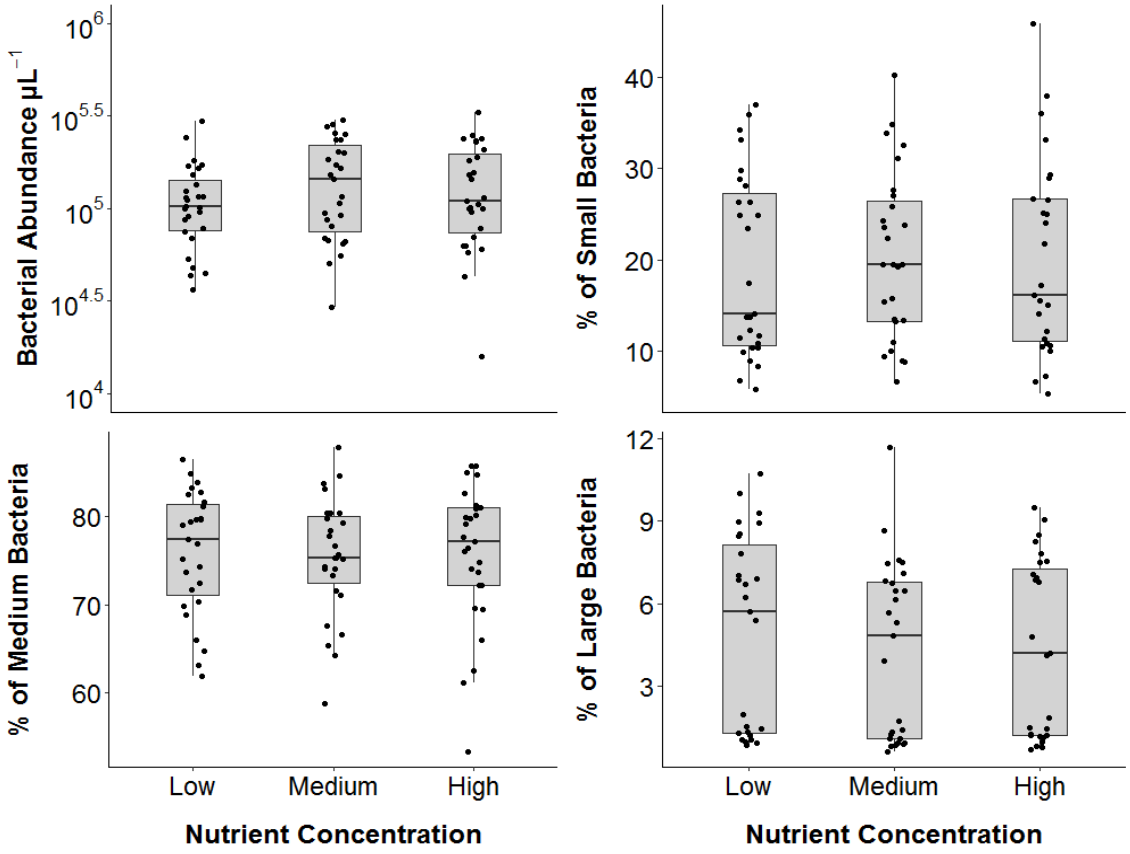
Experiment 1

Treatment	Repl. Block 1	Repl. Block 2	Repl. Block 3	Repl. Block 4
No consumer + low nutrient	2	2	2	3
No consumer + med. nutrient	2	2	2	3
No consumer + high nutrient	2	2	2	3
N. fontanus + low nutrient	2	2	2	3
N. fontanus + med. nutrient	2	2	2	3
N. fontanus + high nutrient	2	2	2	3
P. cavaticus + low nutrient	2	2	2	3
P. cavaticus + med. nutrient	2	2	2	3
P. cavaticus + high nutrient	2	2	2	3
				Total 81 microcosms

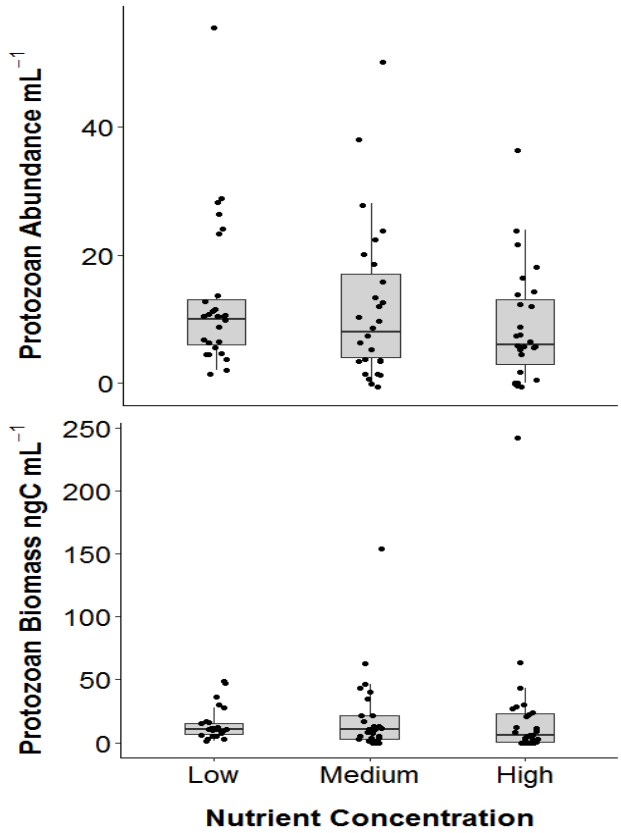
Experiment 2

Treatment	Replicates
No consumer	10
N. kochianus low density	10
N. kochianus high density	10
	Total 30 microcosms

Results & Discussion:



Appendix 5.2.2: The effect of different nutrient concentrations (low, medium, high) on bacterial abundances (cells μl^{-1}, top left), the % of small bacteria (top right), the % of medium bacteria (bottom left) and the % of large bacteria (bottom right) in experiment 1. Boxplots, with individual data points superimposed, summarise data from four experimental blocks.



Appendix 5.2.3: The effect of different nutrient concentrations (low, medium, high) on protozoan abundances (ind. mL^{-1}, top) and protozoan biomass (ngC mL^{-1}, bottom) in experiment 1. Boxplots, with individual data points superimposed, summarise data from four experimental blocks.

In the initial ANOVA models nutrients did not have significant effects on bacterial or protozoan response variables ($P > 0.05$; see figures above) and were therefore excluded from further analysis. The nutrient addition did not influence bacterial abundance or size classes (Appendix 5.2.2), nor was any effect propagated up the food web to protozoans (Appendix 5.2.3). The absence of a bottom-up effect in this study may be because only one nutrient (N) was provided to the bacteria, whereas further nutrients (e.g. phosphorus, P) are often needed for bacteria to respond. Several studies have found that nutrients are often co-limiting bacterial assemblages in various aquatic systems (e.g. Graneli et al., 2004; Mills et al., 2008; Obernosterer et al., 2015). An alternative, albeit less likely, hypothesis is that groundwater microbes did not respond because the nutrient concentrations were too low to elicit reproduction in the bacteria. Many organisms in groundwater have adapted to their low-energy environment by evolving lower

reproductive and metabolic activity rates (Gibert et al., 1994; Gibert & Deharveng, 2002). In other groundwater experiments microbes did respond to increased nutrient concentrations (e.g. Foulquier et al., 2010, 2011), so it is not clear whether reduced metabolic activity played a role in this experiment. In these studies, three concentrations of easily degradable DOC sodium acetate were used, which resulted in significant increases of bacterial biomass and growth. The positive effect of this type of DOC was also corroborated by other studies (Molz et al., 1986; Findlay et al., 2003), indicating that it perhaps has higher bioavailability and is more easily processed by groundwater microbes than the NH_4NO_3 used in this study.

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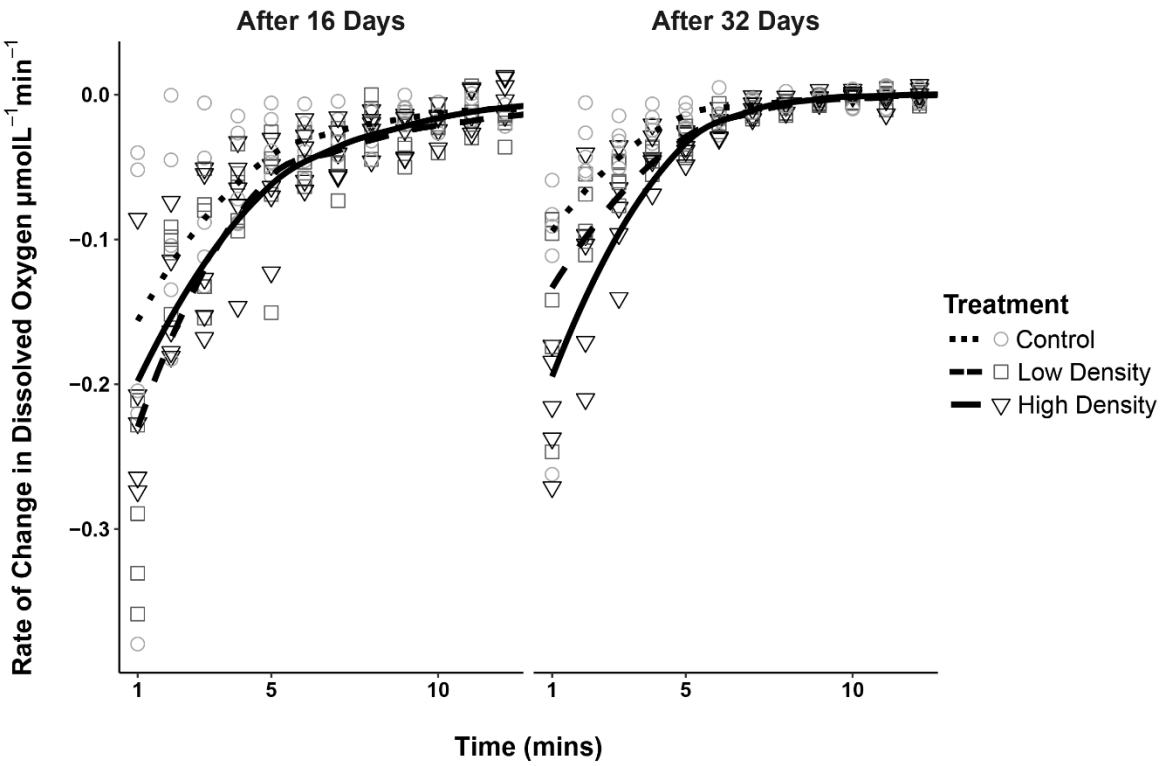
Appendix 5.3: Methodology, results, brief discussion and references for the respiration rates measured in experiment 2.

Methodology:

To determine respiration rates in different treatments, one small tile was removed from five randomly selected replicates of each treatment after 16 and 32 days, respectively. Each tile was immersed in autoclaved water in a tightly sealed 50 ml glass respiration chamber. The chamber was then placed in a dark water bath at a constant temperature of 11 °C. An oxygen microoptode (Unisense, Aarhus, Denmark) was used to measure the change in oxygen concentrations over a 30-minute period. SensorTrace software from the same company was used to obtain temperature-corrected oxygen data. For subsequent analysis the first 15 minutes of data were removed, to allow for an initial 'calibration period'. Techniques to measure changes in oxygen concentrations are a widely used proxy for community respiration and activity levels of microscopic organisms (Gatti et al., 2002; Moodley et al., 2008; Perkins et al., 2012).

Results & Discussion:



Appendix 5.3.1: Rate of change in dissolved oxygen concentrations for different grazer treatments in experiment 2 after 16 days (left) and at the end of the experiment (right) with Loess curves fitted as lines of best fit. While there were no significant differences between the treatments after 16 days, assemblages exposed to high grazing densities had considerably higher respiration rates than the control after 32 days.

Overall, it appears that high grazing pressure increases community activity towards the end of the 32-day experimental period. This corroborates the increases in protozoan abundance and morphotype diversity, and the changes in bacterial community composition observed in the chapter. These data were not used in the submitted version of the manuscript, because only five replicates were measured and the effect was only marginally significant.

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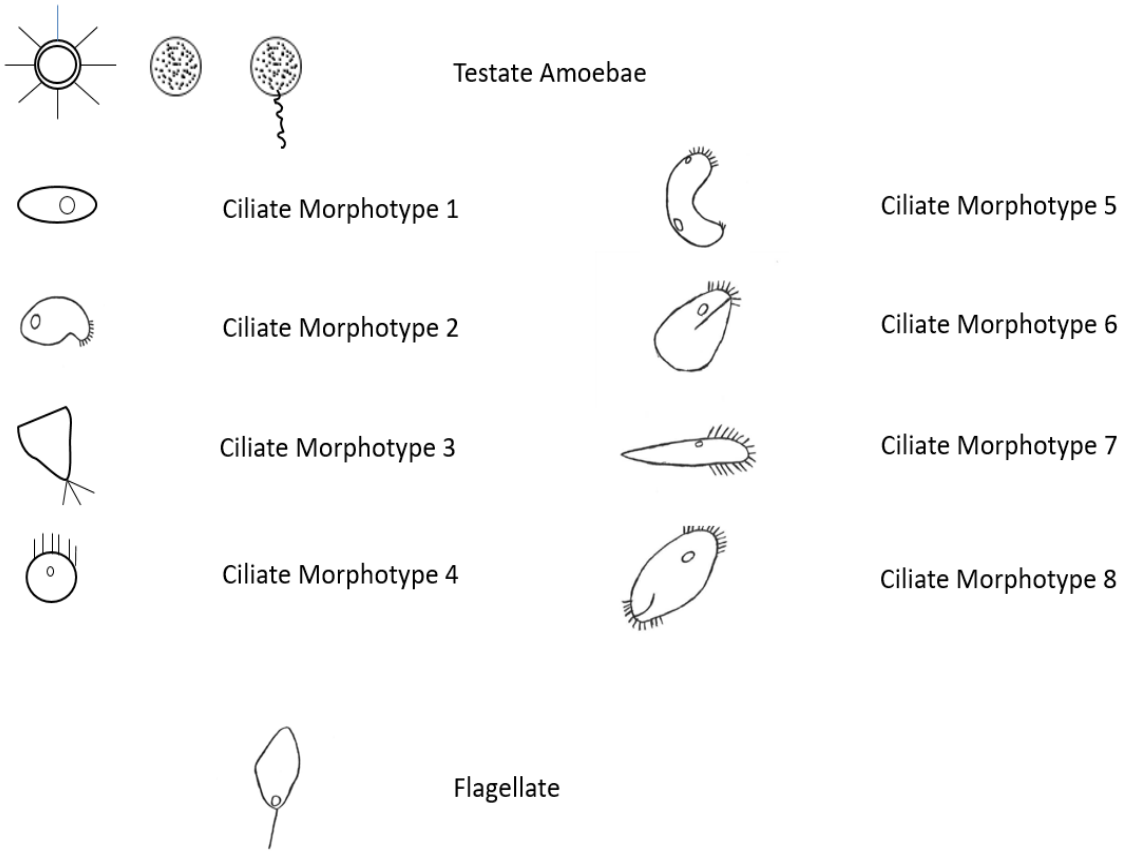
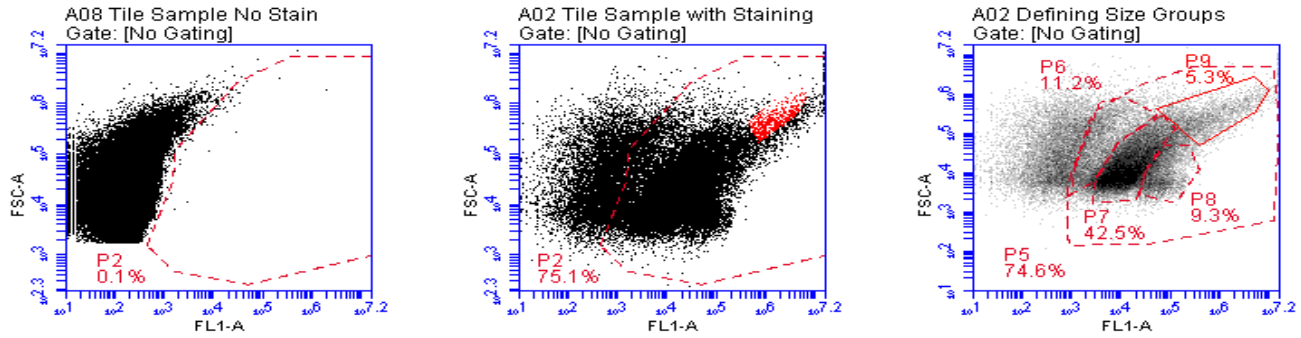
Appendix 5.4: Bacterial and protozoan response variables analysed in experiments 1 and 2. (*) Bacteria were detached with a toothbrush in experiment 1 and pipetted directly from the tile in experiment 2. (**) Respiration is a community-level response that acts as a proxy for bacterial and protozoan activity and population levels.

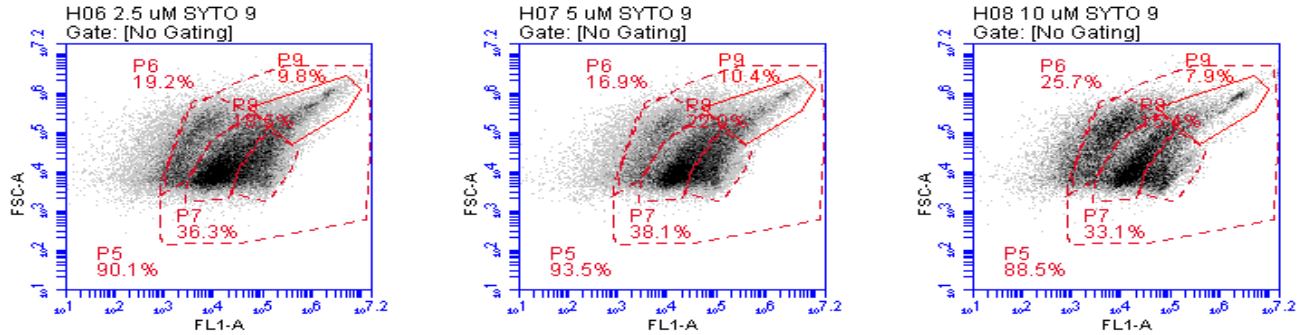
	Bacterial Parameters	Protozoan Parameters		Bacterial Parameters	Days on which measured	Protozoan Parameters	Days on which measured
Experiment 1	Abundance (*)	Abundance	**Experiment 2**	Abundance (*)	2, 5 11, 16, 23, 32 (all bacterial parameters)	Abundance	2, 5 11, 16, 23, 32 (all protozoan parameters)
	Proportion of small-size bacteria	Biomass (carbon content)		Proportion of small-size bacteria		Biomass (carbon content)	
	Proportion of medium-sized bacteria			Proportion of medium-sized bacteria		Morphotype Diversity	
	Proportion of large-size bacteria			Proportion of large-sized bacteria			
				Abundance from exclusion tiles			
				Size proportion from exclusion tiles			
						Respiration (**)	16, 32



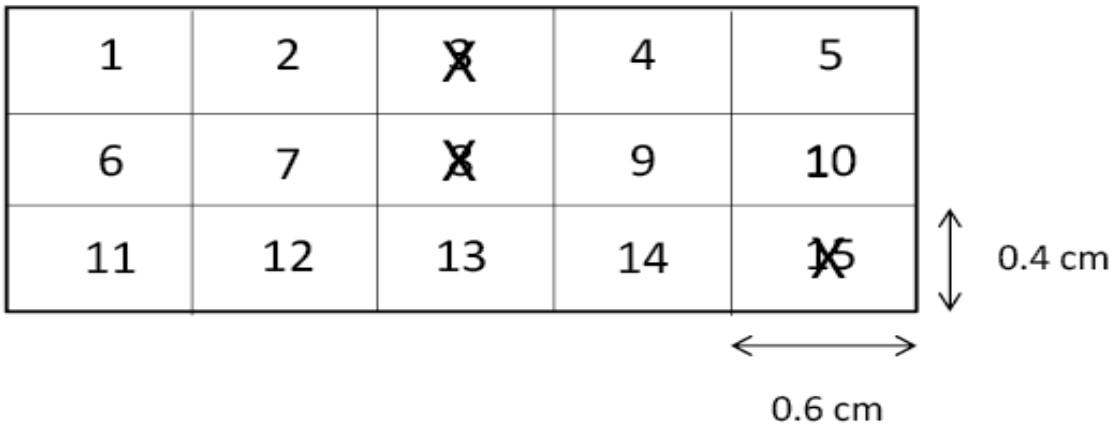
Appendix 5.5: Diagram of the 10 protozoan morphotypes that individuals were assigned to. Morphotypes were identified and drawn at x400 magnification using a CX Olympus microscope. The sketches are not to scale.



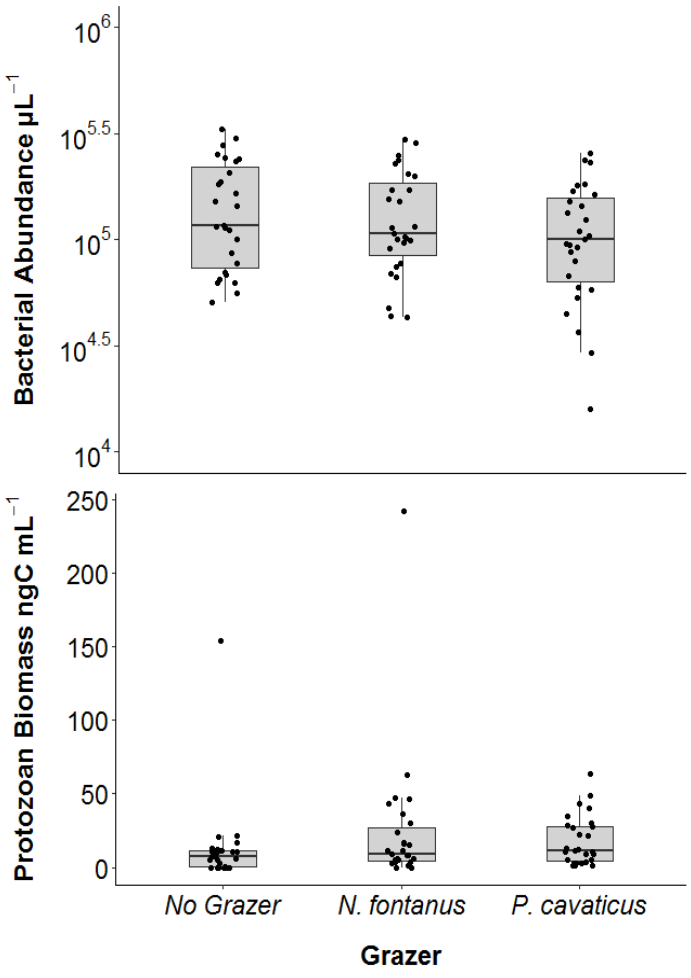
Appendix 5.6: BD6 flow cytometer plots showing an unstained sample (left), a stained sample (middle) and the definition of bacterial size groups (small, medium, large) (right). The bacterial gate in (b) was drawn to define the scatter and fluorescence properties of bacteria in the samples.



Appendix 5.7: BD6 flow cytometer plots showing samples stained with 2.5 µM SYTO 9 (left), 5 µM SYTO 9 (middle) and 10 µM SYTO 9 (right). Only 1 of 3 replicates for each dilution is shown. Staining with 5 µM consistently marked the highest number of bacteria and resulted in a clear distinction of bacterial size groups. This concentration was therefore chosen for the experiments.



Appendix 5.8: Schematic diagram showing the gridded tiles used for bacterial sampling in experiment 2. Each square was 0.6 x 0.4 cm. Squares were chosen randomly each time and previously sampled squares were marked with an X to avoid resampling.



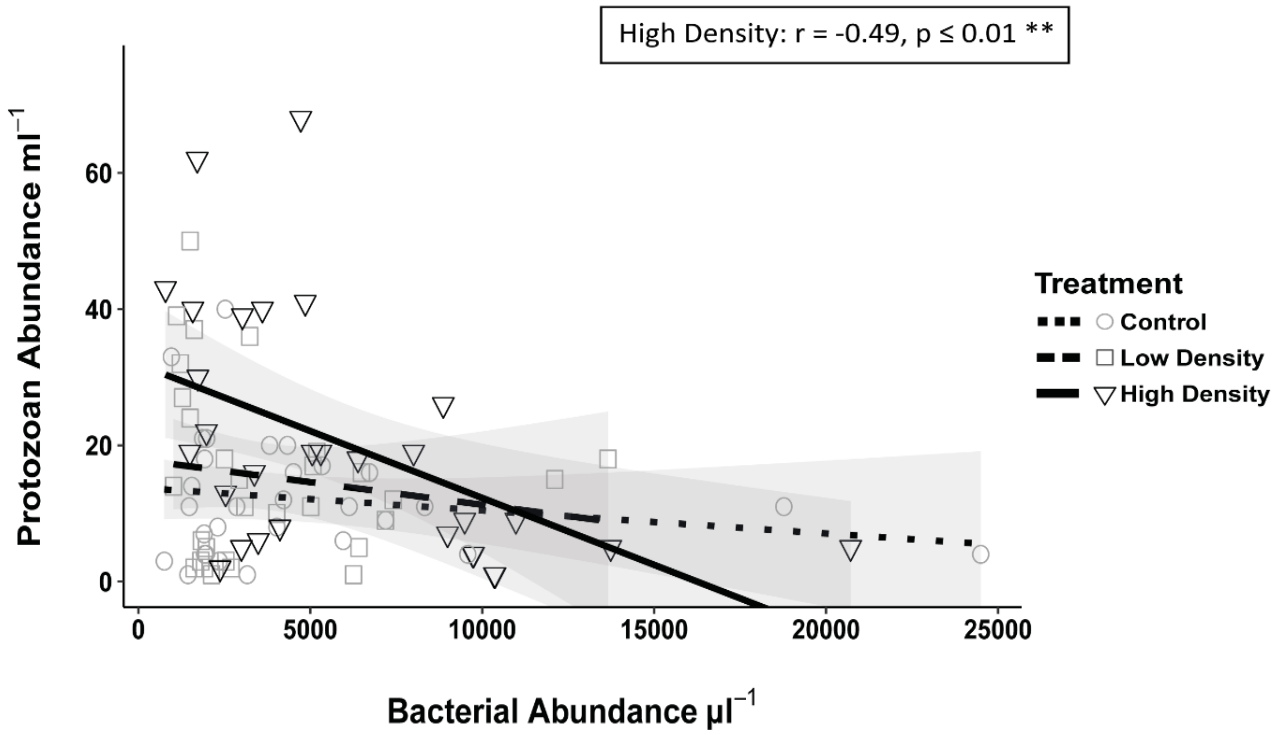
Appendix 5.9: The effect of a no grazing control and single individuals of *N. fontanus* and *P.* cavaticus on bacterial abundances (cells μL^{-1}) and protozoan biomass (ngC mL^{-1}) in experiment 1. Boxplots, with individual data points superimposed, summarise data from four experimental blocks. Grazing of both stygobites resulted in slightly reduced bacterial abundance. Furthermore, protozoans in grazed conditions had considerably higher mean biomass.

Appendix 5.10: Bacterial and protozoan response variables collected in experiment 2 showing the mean and standard error (SE) for the different grazing density treatments and days. For NA values no data was obtained on the given day.

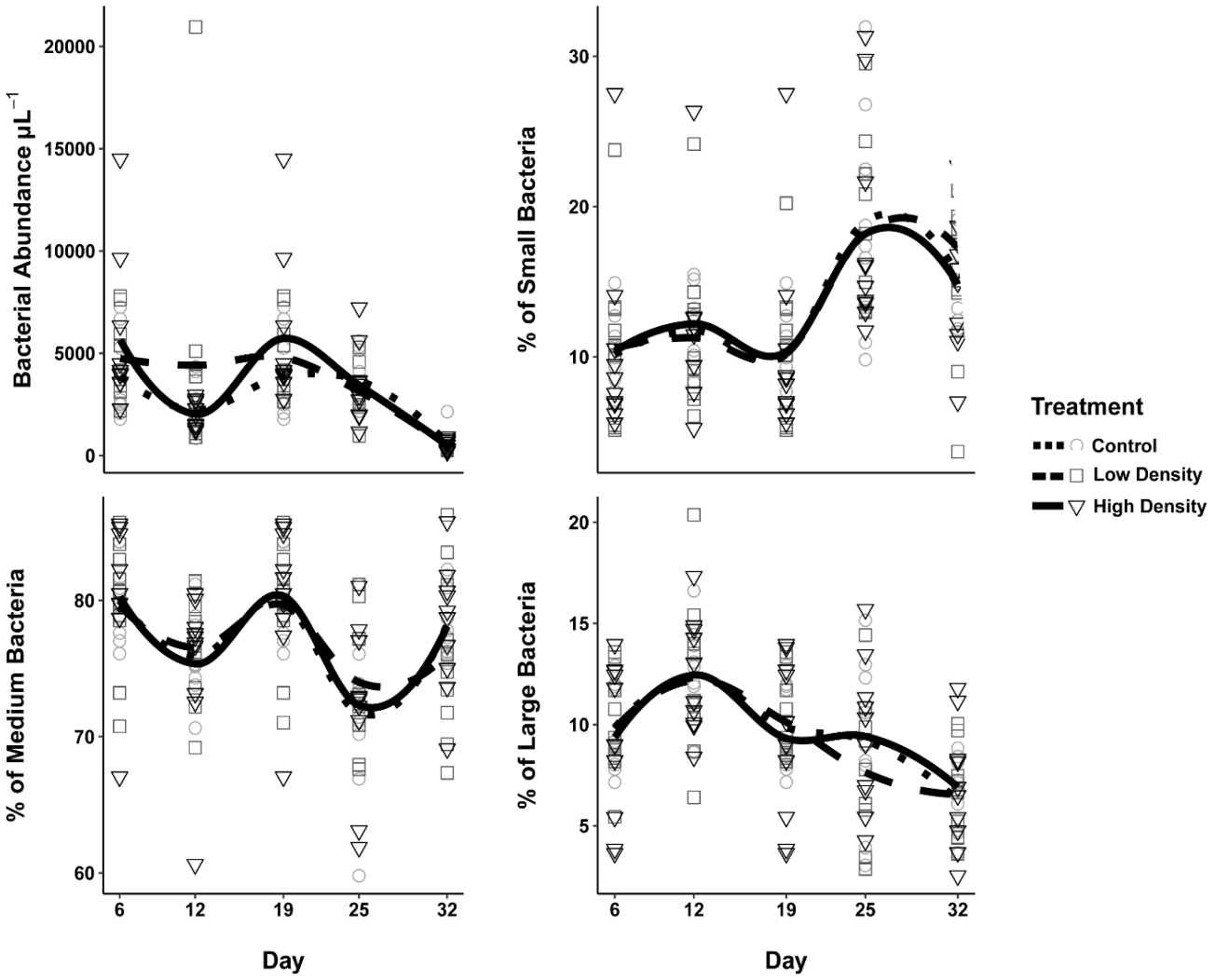
Response	Treatment	Day 2	Day 3	Day 5	Day 9	Day 11	Day 16	Day 18	Day 23	Day 27	Day 32
Bacterial Abundance	ND	1808 (238.9)	2917 (334.3)	5823 (2210.6)	14584 (10391.6)	5531 (500.5)	8247 (1295.7)	3594 (409.2)	2570 (727.1)	4089 (404.5)	2249 (428.7)
	LD	2304 (473.1)	3401 (258.3)	4999 (1662)	26949 (21191)	5472 (503.3)	7869 (976.1)	6349 (2878.3)	2675 (608.3)	6137 (1052.1)	2190 (277.1)
	HD	13850 (3552.4)	4557 (330.9)	5160 (1767)	10064 (4343.4)	6785 (919.7)	6778 (862.2)	4716 (1323.4)	2273 (470.2)	5591 (697.8)	2379 (299.1)
Bacteria / Particle	ND	0.32 (0.06)	0.43 (0.04)	0.36 (0.06)	0.41 (0.08)	0.41 (0.03)	0.42 (0.03)	0.46 (0.02)	0.44 (0.03)	0.85 (0.01)	0.25 (0.04)
	LD	0.41 (0.05)	0.4 (0.04)	0.37 (0.06)	0.38 (0.09)	0.42 (0.03)	0.45 (0.03)	0.54 (0.05)	0.44 (0.04)	0.9 (0.01)	0.37 (0.09)
	HD	0.49 (0.02)	0.44 (0.03)	0.52 (0.06)	0.5 (0.1)	0.49 (0.03)	0.48 (0.06)	0.5 (0.05)	0.52 (0.05)	0.9 (0.01)	0.4 (0.07)
Protozoan Abundance	ND	4 (1.14)	NA	4.6 (1.69)	NA	12.8 (1.66)	11.4 (2.34)	NA	19.6 (4.21)	NA	20.6 (5.1)
	LD	2.4 (0.93)	NA	4.8 (1.62)	NA	13.2 (2.01)	13.2 (2.27)	NA	31.2 (2.52)	NA	27.2 (7.3)
	HD	3 (0.84)	NA	6.2 (1.83)	NA	13.8 (3.73)	16.2 (1.88)	NA	46.6 (8.29)	NA	34.2 (4.37)
Protozoan Biomass	ND	0.0029 (0.001)	NA	0.0022 (0.0001)	NA	0.0092 (0.0028)	0.0088 (0.0022)	NA	0.0143 (0.0042)	NA	0.0051 (0.0001)
	LD	0.0013 (0.0001)	NA	0.0031 (0.0011)	NA	0.0122 (0.0049)	0.0077 (0.0021)	NA	0.012 (0.001)	NA	0.0084 (0.002)
	HD	0.013 (0.0082)	NA	0.0021 (0.0001)	NA	0.011 (0.0055)	0.008 (0.0001)	NA	0.024 (0.0045)	NA	0.013 (0.0028)
No. of Protozoan Morphotypes	ND	1.6 (0.4)	NA	2 (0.32)	NA	3 (0.32)	3.2 (0.37)	NA	3.4 (0.4)	NA	2.4 (0.24)
	LD	1.4 (0.4)	NA	2.2 (0.37)	NA	3.6 (0.4)	3.2 (0.37)	NA	3 (0.32)	NA	2.6 (0.24)
	HD	1.6 (0.4)	NA	2.6 (0.24)	NA	3.4 (0.24)	3 (0.32)	NA	3.6 (0.51)	NA	4.4 (0.4)

Appendix 5.11: Results of post-hoc paired dependent t-tests showing significant comparisons for the protozoan and bacterial response variables. P-values are Bonferroni-corrected.

Biota	Response Variable	Effect	Comparison	t	P
Protozoa	Abundance	Treatment	No Density – High Density	-2.1	< 0.05 *
		Day	Day 5 – Day 11	-5.54	< 0.001 ***
		Day	Day 16 – Day 23	-5.22	< 0.001 ***
		Treatment : Day	No Density Day 23 – High Density Day 23	-2.2	< 0.001 ***
	Biomass	Day	Day 5 – Day 11	-5.12	< 0.001 ***
		Day	Day 16 – Day 23	-4.67	< 0.001 ***
	No. of Morphotypes	Treatment	No Density – High Density	-2	< 0.05 *
		Day	Day 5 – Day 11	-4.83	< 0.001 ***
Bacteria	Abundance	Day	Day 23 – Day 27	-5.1	< 0.001 ***
		Day	Day 27 – Day 32	6.27	< 0.001 ***
	Bacteria / Particle	Day	Day 23 – Day 27	-17.85	< 0.001 ***
		Day	Day 27 – Day 32	13.83	< 0.001 ***
	Small Bacteria	Treatment : Day	No Density Day 2 – High Density Day 2	-10.98	< 0.001 ***
		Treatment : Day	Low Density Day 2 – High Density Day 2	-17.49	< 0.001 ***
	Medium Bacteria	Treatment : Day	No Density Day 2 – High Density Day 2	8.5	< 0.001 ***
		Treatment : Day	Low Density Day 2 – High Density Day 2	15.6	< 0.001 ***
	Large Bacteria	Treatment : Day	No Density Day 2 – High Density Day 2	8.06	< 0.001 ***
		Treatment : Day	Low Density Day 2 – High Density Day 2	7.96	< 0.001 ***



Appendix 5.12: Correlation showing the negative relationship between bacterial abundance (µL^{-1}) and protozoan abundance (mL^{-1}) in experiment 2. This relationship is only significant (P < 0.01) in the high grazing density treatment, supporting the theory that protozoans have increased 'access' to bacteria when stygobites are abundant.



Appendix 5.13**:** The effect of control and different grazing density treatments (low and high) of *N. kochianus* on bacterial abundance (ind. μl^{-1}, top left), the % small bacteria (top right), the % of medium bacteria (bottom left) and the % of large bacteria (bottom right) over time on tiles excluded from grazing by mesh nets in experiment 2. Different grazing treatments are symbolised by dotted (control), dashed (low density) and solid (high density) lines. Bacterial responses were sampled on six occasions (on day 2, 5, 11, 16, 23 and 32). In contrast to tiles accessible for grazing, stygobites had no effects on bacterial community composition on exclusion tiles, providing a suitable control for the effects seen in the experiment.

Chapter 6 - General discussion

Explaining the observed distributions of biological communities across habitats is one of the main challenges in ecological research (Poff, 1997). Although individual factors have long been known to influence community compositions, ecologists have only recently acknowledged that biotic patterns are the result of multiple interacting forces, operating across a wide range of spatial and temporal scales (Allen & Starr, 1982; Hilborn & Stearns, 1982; Hahn, 2009; Stoch & Galassi, 2010). Poff (1997) identified multiple hierarchical controls (broad basin, reach, channel unit and microhabitat scales) that act as filters and shape biotic assemblages in rivers. Only species with appropriate functional attributes or adaptive behaviours pass a filter and are present in the next hierarchical scale. While this concept was initially proposed for rivers, it is applicable to a wide range of ecosystems and organisms, for which similar filters are present (McGill et al., 2006; Westoby & Wright, 2006; Green et al., 2008). The integrative study of such different scales is challenging in all ecosystems; and particularly so in the groundwater environment, where many processes are hidden from the human eye, access points are rare and knowledge of ecological species traits and habitat requirements are limited (Gibert & Culver, 2009). Consequently, the field of groundwater ecology lags behind that of surface water ecology, and has only in the last two decades moved towards integrative explanations of the observed ecological patterns (Larned, 2012).

The acknowledgement of multi-scale controls on groundwater ecosystems (Gibert et al., 2009) culminated in the PASCALIS project, a trans-national investigation of the controls of stygobite distributions. Analogous to Poff (1997), a stratified sampling design was employed to capture environmental heterogeneity at different spatial scales (Galassi

et al., 2009), including region, hydrogeographic basin, aquifer type (geology) and hydrogeological zone (micro-habitat). The development of more complex theoretical frameworks and sampling designs has provided increased explanatory power for groundwater studies and, overall, has greatly advanced the field of groundwater ecology.

Geology (or aquifer type) is an important determinant of groundwater communities (Robertson et al., 2009; Dole-Olivier et al., 2009; Galassi et al., 2009; Martin et al., 2009; Johns et al., 2015). A major reason for this is that geology controls further abiotic parameters, which in turn influence faunal assemblages at all spatial scales (small-, mid- and large-scale) (Fig. 6.1; and see Fig. 1.2 in introduction). These parameters include small-scale variation in hydrochemistry at individual sites, the mid-scale availability of physical habitat space in the geologies and the large-scale availability of connected dispersal pathways that reinforce processes operating in deep geological time (e.g. glaciations, marine transgressions or regressions, etc.). This thesis demonstrates the influence (positive and negative) of geology and its associated heterogeneity on groundwater communities across multiple spatial scales. To achieve this, considerable geological detail was included in chapters two, three and four of this thesis.

The geo-habitat typology in chapter two is a new way of characterising groundwater habitats and is augmented with habitat quality scores that account for habitat heterogeneity, which may influence faunal communities. An ecological assessment of four important UK aquifers was undertaken in chapter three, consulting hydrogeological information from aquifer properties manuals (Allen et al., 1997; Jones et al., 2000) to explain the observed distributions. The meta-analysis of the UK stygobite records investigated how geology affects species distributions at a national scale, in the

context of my new typology. To further the discussion on the importance of stygobites in groundwater ecosystems and their potential involvement in the provision of ecosystem services (e.g. bioremediation, maintaining water quality), manipulative experiments were undertaken to assess their effects in food webs as part of chapter five. There is a strong overarching link between the chapters and their results are mutually informative, often building on each other. In summary, my thesis increases our understanding of how geological heterogeneity influences groundwater ecosystems (chapters two – four) and the role of stygobites in their ecosystems (chapter five).

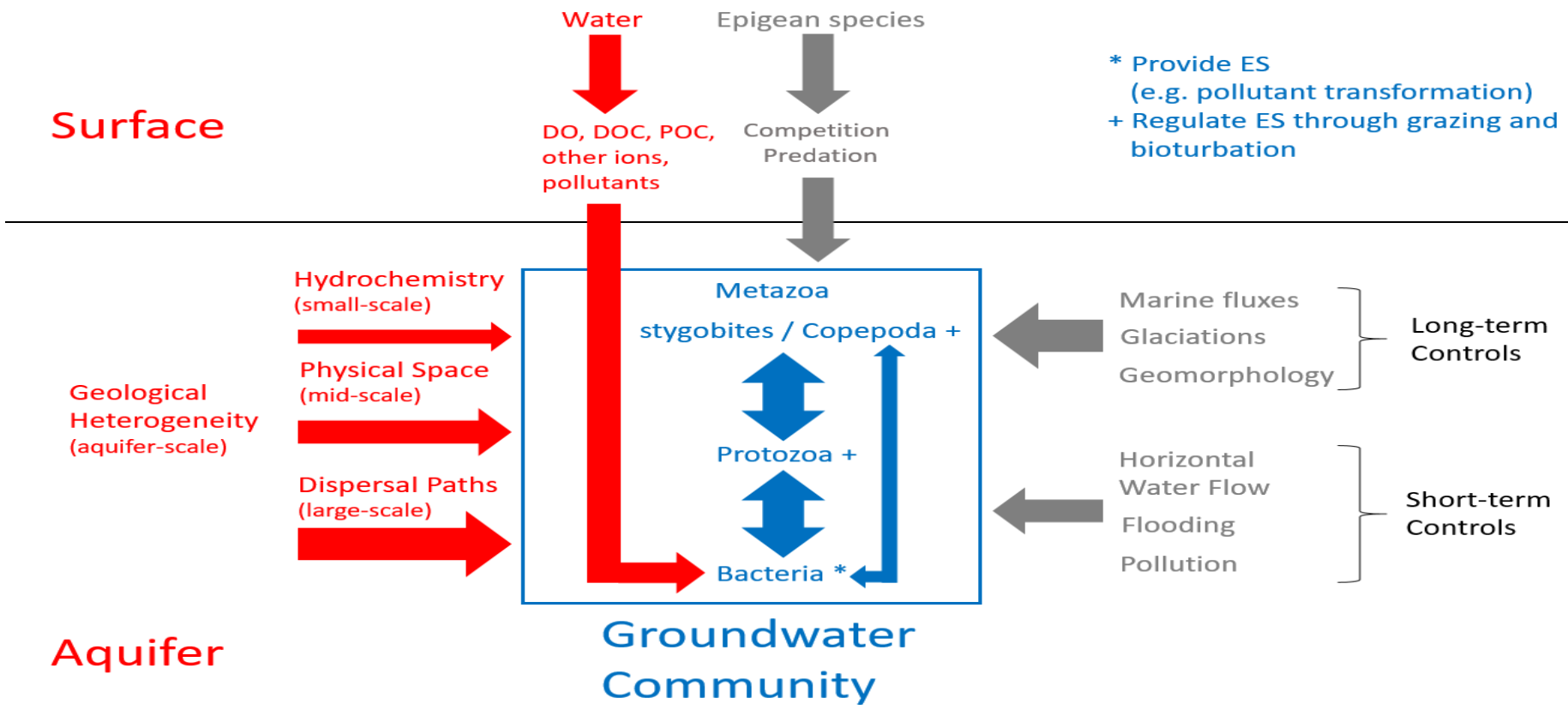


Fig. 6.1: Conceptual diagram illustrating the major linkages (or controls) between abiotic and biotic components in groundwater. The thickness of the arrows indicates the interaction strength between components. Single red arrows indicate the abiotic controls, while the blue box and two-way arrows indicate the biotic interactions studied. Geology, an aquifer-scale control, influences abiotic characteristics at all spatial scales, which in turn control groundwater communities. Other important long-term and short-term controls (e.g. glaciations, pollution) that were not directly studied are marked in grey. ES = ecosystem services

6.1 Geological heterogeneity influences stygobites at all spatial scales

Geology clearly influences stygobite distribution across all spatial scales. This has been attributed to its influence on habitat characteristics across multiple spatial scales (Fig. 6.1). At the large-scale, the geo-habitat typology developed in chapter two indicates that there are a wide range of habitats in England and Wales with significant differences in DO, DOC, nitrate and calcium, most likely important to stygobites (Rukke, 2002; Datry et al., 2005; Hahn, 2006; Dole-Olivier et al., 2009). Furthermore, the quality scores indicate that poor habitats dominate in most geographic areas, which are likely to harbour reduced biodiversity and restrict dispersal. This is corroborated by the large-scale distribution of most species in chapter four, which showed that most species predominantly occur in karstic geo-habitats (e.g. Chalk, Moderately Karstic and Highly Karstic Limestone).

Generally, all stygobite species in England and Wales have relatively large distribution ranges and occur in multiple geo-habitats, compared to mainland Europe where many species are only found in a single location (e.g. Dole-Olivier et al., 2009; Galassi et al., 2009). Due to this habitat generalism, geological controls may most suitably be defined as preferential occurrence in a particular geo-habitat over others; i.e. occurrence that is statistically different from chance. Similar to previous studies (Castellarini et al., 2007; Hahn & Fuchs, 2009; Johns et al., 2015), chapter four provided evidence that geology controls species assemblages at the mid-scale. Such geological controls were evident for several species, including *N. kochianus* and *N. fontanus*, which occurred preferentially in the Chalk. Some geo-habitats (e.g. Highly Karstic Limestone, Chalk, Mixed Sandstone) also harboured higher biodiversity than others (e.g. Igneous Rock, Metamorphic Rock), providing additional indication for a geological control at the

mid-scale. Furthermore, both the Mudstones & Siltstones (geo-habitat, chapter four) and the Lower Greensand (aquifer, chapter three) were found to be poor habitats, with the latter not containing stygobites in any of the samples.

At the small-scale, the within-habitat variability of all proposed geo-habitats in chapter two remained extremely high, even though the traditional groupings of porous, fractured and karstic rocks (e.g. Hahn & Fuchs, 2009) were further refined to account for more geological detail. All geo-habitats have many 'good' and 'bad' habitat patches in hydrogeological (e.g. transmissivity) and hydrochemical (e.g. DO, DOC, nitrate and calcium) terms, most likely determined by geology (Fig. 6.1). For example, the occasional stygobite records in the Mudstones & Siltstones, which may be related to the availability of such good habitat patches, also indicate how geology controls species distributions at the small-scale and mid-scale.

To account for this within-habitat heterogeneity a system of habitat quality scores was developed (see section 6.2). The variability of resources in geo-habitats was reflected in the heterogeneous distribution of stygobites in chapter four. As in former studies (e.g. Dole-Olivier et al., 1997; Dumas, 2002; Johns et al., 2015), many sites appeared not to contain stygobites, resulting in the patchy distribution of species (Datry et al., 2005; Dole-Olivier et al., 2009).

Small-scale site variability explained the highest proportion of copepod biomass and abundance in chapter three, superseding the effects of all other environmental variables, including general aquifer geology. Similar effects are also reported in previous studies (Hahn, 2006; Humphreys, 2008; Hancock & Boulton, 2009). One of the possible explanations for this is that not enough geological detail was captured at the aquifer

level, because both lithology and permeability are highly variable within single aquifers (Allen et al., 1997; Jones et al., 2000). Unexplained geological heterogeneities (e.g. interception of very permeable voids) may be the source of the significant between-site differences, possibly affecting nutrient availability (and therefore biomass and abundance) at individual sites (Fig. 6.1). However, non-geological site-specific factors (e.g. site open / sealed) probably also explain a high proportion of the variance in copepod biomass and abundance.

Overall, the chapters in this thesis are a collection of evidence for the role of geological heterogeneity and complement those studies that have already discussed its importance for groundwater assemblages (e.g. Gibert et al., 1994; Hahn, 2006; Deharveng et al., 2009; Galassi et al., 2009; Stoch et al., 2009; Griebler et al., 2010; Sorensen et al., 2013).

6.2 The new geo-habitat typology

During the trans-national research programme PASCALIS (Galassi et al., 2009; Gibert et al., 2009), groundwater scientists have started to use hierarchical sampling programmes to capture environmental controls across the different spatial scales (Fig. 6.1). Surprisingly, although hydrogeological aquifer type has been identified as one of the most influential controls on groundwater ecosystems (Gibert, 2001; Fuchs et al., 2006; Hahn, 2009), few previous studies have gone beyond the traditional division of karstic, fractured and porous rocks (see Cornu et al., 2013; Johns et al., 2015). The typology that I developed for England and Wales considered lithological detail at the 1:50k scale to

define 11 distinct geo-habitats. It was then verified by comparing the hydrogeological and hydrochemical characteristics of habitats in chapter two, and their biota in chapter four.

The abiotic and biotic patterns in geo-habitats confirmed that the classification approach adopted in chapter two captures lithological detail that is important to stygobites. The hydrochemical conditions differed significantly between geo-habitats. For example, while some habitats had very low mean DO (Mildly Karstic Limestone, Small-Pore Unconsolidated), others had very high mean DO (Igneous Rock, Chalk), and similar patterns were evident for all other abiotic parameters.

The stygobite communities also differed considerably between geo-habitats. This finding was particularly important for fractured rocks, because recent studies have identified the need for a better ecological understanding of these habitats (Hahn & Fuchs, 2009; Larned, 2012). A high stygobite frequency of occurrence was found in Igneous Rock (chapter four; Johns et al., 2015), highlighting that fractured rocks may be important groundwater habitats. Furthermore, fractured habitats such as Igneous Rock and Metamorphic Rock are important because they harbour *N.* glenniei, the only endemic (Knight, 2009) and most narrowly distributed species in England and Wales. Mudstones & Siltstones harboured a higher than expected species diversity (four species compared to two species in Igneous Rock), but at much lower frequencies of occurrence. These results suggest that my habitat typology is suitable for national-scale studies, and more appropriate than coarser classifications. Despite clear differences in their significance for groundwater communities, Igneous Rock, Metamorphic Rock and Mudstones & Siltstones would have all been grouped as fractured habitats in previous studies (e.g. Hahn, 2009; Hahn & Fuchs, 2009). Therefore, my new typology illustrates that broad groupings

(karstic, porous, fractured) may not capture sufficient geological detail to explain stygobite distributions at the national scale.

However, the high within-habitat heterogeneity of abiotic and biotic characteristics (chapters two and four) shows that for regional and local studies even more geological detail needs to be considered to assess stygobite distributions. For example, the relatively high species diversity in Mudstones & Siltstones was most likely due to very infrequent patches of limestone or sandstone beds (see Jones et al., 2000), which may act as high-quality habitat islands in an otherwise unsuitable geo-habitat. The geological resolution I used in my typology was not sufficient to distinguish these different mudstone lithologies. It is likely that some of the stygobite-containing strata within the Mudstones & Siltstones geo-habitat, would be included in another geo-habitat in a typology that considers the proportions of different lithologies at individual sites. Overall, this shows that habitat typologies need to be developed with respect to the specific scale they are to be used for. It is very difficult to devise a national-scale habitat classification that incorporates sufficient geological detail to explain stygobite distributions in the highly heterogeneous fractured habitats. My study suggests that further sampling of fractured habitats in local and regional studies within more detailed geological frameworks is needed to understand their ecology (see section 6.7).

To incorporate this within-habitat complexity into my analysis, I developed a quantitative method that considers habitat variability and enables an assessment of how suitable geo-habitats are for groundwater ecosystems in terms of species diversity and frequency of occurrence. Selecting the parameters that are influencing groundwater communities (e.g. transmissivity, DO, DOC, nitrate, calcium) was the initial step, as

variation in these is most likely to determine the complexity and composition of resident communities (Mösslacher, 1998, 2000; Rukke, 2002; Scarsbrook & Fenwick, 2003; Datry et al., 2005; Paran et al., 2005). The second, and perhaps most important step, was to incorporate a variability term for each of the parameters into the mathematical equation I used to calculate habitat quality. To accomplish this, parameter thresholds were extracted from the literature and the ratio of suitable to unsuitable patches for each parameter was calculated. Groundwater habitats are highly heterogeneous (chapter two; Larned, 2012; more) and this variability controls groundwater communities across all spatial scales (see section 6.1; Galassi et al., 2009; Gibert et al., 2009). In his review, Larned (2012) highlights the importance of small-scale heterogeneities in preferential flowpaths and lithofacies for local permeability, hydrochemistry and, ultimately, faunal communities. The habitat quality scoring system I developed in this thesis is an advance because it incorporates the small-scale heterogeneity of abiotic parameters highlighted by Larned (2012), and consequently enables a more detailed understanding of ecosystem distribution controls.

In most instances, the proposed quality of geo-habitats was reflected by stygobite communities, with higher quality habitats generally supporting higher stygobite diversities and frequencies of occurrence. Quality scores developed in the framework of similar typologies may be used to predict species richness and occurrence in aquifers. Predicting biodiversity in complex habitats is very important, because sampling over large spatial scales is expensive and time-consuming to undertake (Stoch et al., 2009). Although indicator species are sometimes used as biodiversity predictors (McGeoch, 1998; Favreau et al., 2006), the habitat quality indices I developed in this thesis may provide a valuable alternative for predicting stygobite diversity and abundance in future studies. Considering

only general aquifer quality in predicting biodiversity is not sufficient, because both Igneous Rock and Mudstones & Siltstones are relatively poor aquifers, but either have areas of suitable water chemistry (e.g. high DO in Igneous Rock, high DOC in Mudstones & Siltstones) and/or harbour stygobites frequently (e.g. Igneous Rock). Furthermore, as highlighted in previous research, stygobites are controlled by multiple hydrochemical parameters (Datry et al., 2005; Hahn, 2006; Dole-Olivier et al., 2009), suggesting that the assessment of single parameters is also not adequate. My habitat quality scores are integrative habitat indices that may enable a more reliable prediction of faunal communities. Furthermore, they are easily adaptable to other regions of the world if relevant hydrochemical data (for example data collected by environmental organisations) are available, possibly enabling a more accurate identification of potentially species-rich habitats. At a smaller scale, the quality scores of single aquifers and the concept of patch quality may indicate target areas for sampling or conservation action. For example, the Large-Pore Unconsolidated geo-habitat has a relatively high quality score (due to its high permeability and suitable water chemistry), indicating it may be a good habitat for stygobites and should be sampled in future studies (see section 6.7).

The thresholds used in chapter two were a first attempt to identify how frequently suitable and unsuitable habitat patches occur within geo-habitats. However, relatively little is known about the hydrochemical requirements and tolerance ranges of stygobites (but see Malard & Hervant, 1999; Brielmann et al., 2009; Di Lorenzo et al., 2015) and therefore the thresholds chosen remain somewhat uncertain (see section 6.7 on future research). Furthermore, stygobites probably do not show a binary response of ‘present’ and ‘absent’ to suitable or unsuitable habitat patches, but are more likely to be a related along a continuum. For example, as demonstrated in Datry et al. (2005), decreasing

nutrient concentrations result in lower stygobite abundances, before nutrients are too low to sustain stygobites altogether. One solution may therefore be to relate the habitat quality scores to species abundance, which is the ecological parameter most likely to reflect this gradual relationship.

6.3 Influence of the glacial legacy in the UK

Most groundwater studies have found that previous glaciations control stygobite communities at the large-scale (Fig. 6.1), with stygobite diversity, frequency of occurrence and abundance being lower in areas that were glaciated (e.g. Juberthie & Decu, 1994; Strayer, 1994; Gibert & Deharveng, 2002; Dole-Olivier et al., 2009; Gibert & Culver, 2005; Gibert et al., 2009; Malard et al., 2009; Martin et al., 2009; Michel et al., 2009; Robertson et al., 2009; Kornobis et al., 2010; Stein et al., 2012; McInerney et al., 2014; but see Culver et al., 2003). One of the conclusions from the PASCALIS study was that stygobite diversity across Europe is predominantly shaped by climate history rather than habitat characteristics (Malard et al., 2009). In the French Jura, species diversity increased significantly with distance from the Wuerm glacier (Dole-Olivier et al., 2009), suggesting that most species were extirpated by the Quaternary permafrost. Similarly, the northern lowlands in Germany are characterised by low stygobite diversity and frequency of occurrence (Hahn & Fuchs, 2009; Stein et al., 2012) due to the long-term effects of Pleistocene ice shields.

Assessing the glacial legacy in the UK requires consideration of the climatic history in the context of species lineage ages. Most UK stygobite species have existed in the UK for substantial periods of time. *N. fontanus* (1 mya), *N. kochianus* (3 mya), *N. aquilex* (6-8

mya) and *N. glenniei* (20 mya) all diverged from the most recent continental ancestor millions of years ago (McInerney et al., 2014). This implies that these species have survived the environmental challenges of at least two glaciations, including the Anglian glaciation approximately 450,000 years ago (Lee et al., 2004) and the Devensian glaciation 10,000 years ago (Walker et al., 1993). Because of the relatively recent occurrence of the Devensian glaciation and the slow dispersal rates of stygobites, current stygobite biodiversity and distribution patterns in the UK are still thought to retain a clear 'Devensian' imprint (Proudlove et al., 2003; Robertson et al., 2009; McInerney et al., 2014). While it is certain that the aforementioned species were present in the UK during repeating glacial-interglacial cycles, their distributions prior to the last glaciation remain unknown, complicating the assessments of glacial impacts.

One of the most fundamental questions is whether glacial extirpations account for the low stygobite diversity in the north and chapter four appears to provide support for this (Weitowitz et al., in prep. a). Except for *A. stammeri* and *N. aquilex*, all species are found widely in southern England and Wales, but are absent in habitats of similar lithology near the Devensian glacial limit. My findings are consistent with those of Robertson et al. (2009), who analysed six aquifers in England and Wales and found that the majority of stygobite records (99 %) come from south of the Devensian glacial limit, concluding that present-day distributions are still shaped by the glacial legacy. One uncertainty in this interpretation is that it is not known whether species were extirpated in northern England or whether they were never present there in the first place. The presence of *A. stammeri*, a bathynellid, in the north may provide an important clue to this question. Given the long time that most other species were present in the UK prior to the last glaciation, it seems unlikely that a bathynellid, known for its slow dispersal (Guil &

Camacho, 2001), would have dispersed to northern England when other species have not. Therefore, it appears that most species would have been present in the north prior to the last glaciation, and were subsequently extirpated.

If species were indeed extirpated during the Devensian glaciation, this effect appears to have extended well into periglacial areas, where permafrost would have been extensive (Downing et al., 1977; Watson & Morgan, 1977; Edmunds & Smedley, 2000), at times up to 100 m thick (Busby et al., 2015). This is indicated by the low stygobite diversity and frequency of occurrence in the northern outcrop of the Jurassic Limestone and the southern outcrop of the Magnesian Limestone, both south of the glacial limit. Model simulations have also shown that the glaciation is likely to have impacted aquifers far beyond the limit of the ice sheet (Boulton et al., 1996), most likely due to the weight of the ice sheet altering the pressure balance in aquifers (Neuzil, 2012) and minimising groundwater circulation and infiltration (Blaser et al., 2010; Alvarado et al., 2011). Furthermore, glaciations may have altered hydrochemical parameters (Piotrowski, 2006; Anderson, 2007) that are potentially important for stygobites.

The second question is whether the distribution patterns within single aquifers allow inferences on the dispersal rates of stygobites in interglacial periods. Chapter three shows that species are not distributed throughout the entire outcrop of Jurassic Limestone (Weitowitz et al., submitted b), a highly suitable (Weitowitz et al., submitted a) and continuous (Allen et al., 1997) aquifer. *N. aquilex*, the species that occurs in both the southern and northern Jurassic Limestone outcrop, and *A. stammeri* may have survived the glaciation in subsurface refugia or could have dispersed to the north since the end of the Devensian glaciation. While the latter seems unlikely for the slow-dispersing *A.*

stammeri, it is a possibility for *N. aquilex*, which is a frequent inhabitant of hyporheic zones that may use such strata to disperse more rapidly (=hyporheic corridor concept, Ward & Palmer, 1994)

Assuming that the presence of *N. aquilex* in the northern Jurassic Limestone is the result of recolonisation, this suggests that most other species have not been able to disperse through a connected aquifer in the last 10,000 years. *P. cavaticus* (113 km distance to *N. aquilex* in northern outcrop), *N. kochianus* (125 km), *M. leruthi* (143 km) and *N. fontanus* (143 km) have not been able to traverse these relatively short distances from their northernmost location in the southern outcrop of the Jurassic Limestone to where *N. aquilex* occurs in its northern outcrop. Several papers have highlighted the low dispersal rates of stygobites (Eberhard et al., 2009; Gibert et al., 2009), and it is now becoming clear that stygobites require long periods of time (millennia) for successful dispersal. Other recent studies have demonstrated high levels of genetic divergence of stygobites in connected habitats (Christman et al., 2005; Culver et al., 2009), supporting the notion of slow dispersal.

Another important emerging characteristic is that the strength of dispersal constraints appears to be species-specific. Some niphargids, such as *N. aquilex* and *N. fontanus*, appear to disperse more efficiently than others. For example, *N. aquilex* has the widest geographic distribution range of all UK species and inhabits most geo-habitats (nine), while *A. stammeri* has only been found in three geo-habitats. Furthermore, *N. fontanus* and *P. cavaticus*, two species preferentially found in karstic habitats, are widely distributed on an east-west axis ranging from south-western Wales to eastern England. However, *N. kochianus*, which co-occurs with these species in the English Chalk, has not

been able to disperse to the cave systems of southern Wales. Overall, glaciation and dispersal controls may act as filters overriding geology (sensu Poff, 1997; Fig. 6.2), limiting distributions of some species (e.g. *N. kochianus*) more than others (e.g. *N. aquilex*).

Assessing the controls of past glaciations and dispersal on stygobite distributions is further complicated by a range of other controls. Biotic interactions, such as competition and predation (Chapman, 1993; Luštrik et al., 2011; Knight & Johns, 2015), hydrochemistry (e.g. Datry et al., 2005) and species-specific tolerances have all been found to influence groundwater communities as well. Therefore, analogous to the concept Poff (1997) proposed for communities in rivers, stygobites appear to be controlled by a series of filters (Fig. 6.2) operating across a range of spatial scales (Fig. 1.1 in introduction). Geology is perhaps the primary one, as suitable habitat is a necessary prerequisite for a species to occur. For example, if pore spaces in a geology are too small to harbour stygobites (e.g. Lower Greensand, chapter three), stygobites cannot be present regardless of whether other criteria of a good habitat are fulfilled; such as long distance from the maximal extent of previous glaciations, the presence of continuous and permeable habitat or suitable water chemistry. If suitable habitat is available, subsequent filters (e.g. glacial history, dispersal, water chemistry and biotic interactions; Fig. 6.2) will then determine species occurrence. While most of these filters are not directly related to physical habitat, they are nonetheless rooted within geological complexity at the mid-scale (Fig. 6.1). Different sets of filters appear to shape stygobite distributions on different axes across England and Wales. On the north-south axis, the glacial history in combination with slow dispersal times appear to be the main filters (Fig. 6.2). On the east-west axis, dispersal constraints appear to be the main filter, possibly in combination with biotic interactions (e.g. for *N. glenniei*; Fig. 6.2). Furthermore, the effects of these filters (e.g.

hydrochemistry) are not always easy to predict. For example, a nutrient increase from low to medium concentrations may increase stygobite abundance and diversity (Datry et al., 2005; Hahn, 2006), but excessively high nutrient concentrations may result in the disappearance of stygobites because they are outcompeted by epigean species (Brunke & Gonser, 1999; Schmidt & Hahn, 2012).

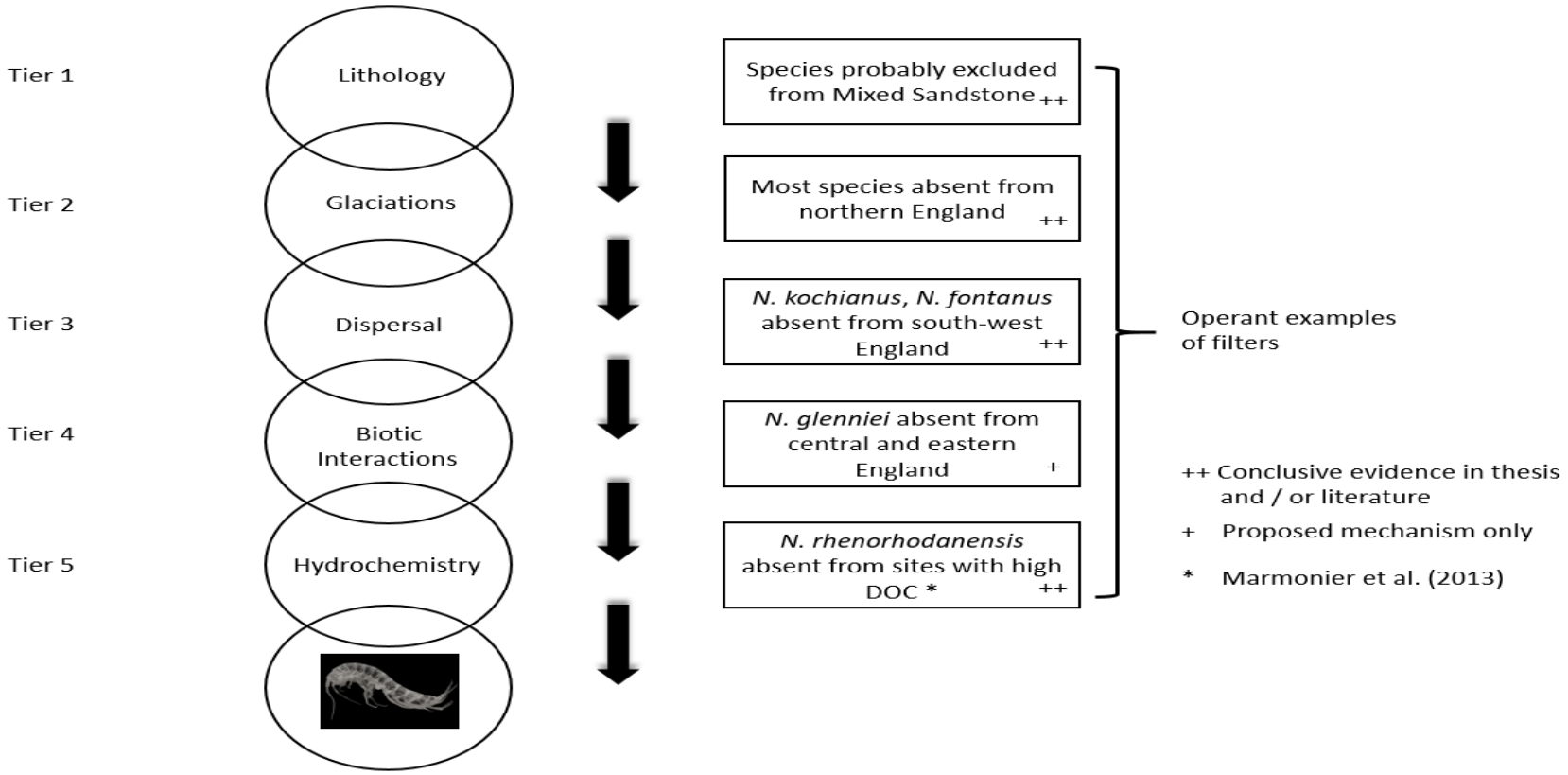


Fig. 6.2: Conceptual diagram summarising the series of filters (circles, sensu Poff, 1997) determining species presence at a given locale. In order to be present at each successive tier, species must pass the respective filters. Examples of each filter in operation are given (boxes) by referring to specific distribution patterns evident in my thesis and the literature. These are discussed in the corresponding sections of the main body of text. This thesis did not investigate hydrochemistry as a species filter, and therefore an example from the literature is given (Marmonier et al., 2013).

6.4 Sampling design may bias species distributions

Factors not related to species ecology, such as sampling design or methodology, may also influence the apparent species distribution patterns. Sampling all habitats evenly is often not feasible, and therefore the number of sampling units needs to be minimised, while incorporating the factors of highest ecological significance into sampling designs (Coddington et al., 1991; Feinsinger, 2001; Hill et al., 2005; Dole-Olivier et al., 2009). In groundwater studies, good examples for these may be the hierarchical filters operating on groundwater ecosystems shown in Fig. 2.

In the past, sampling frameworks for groundwater studies have typically not been statistically designed, mainly due to the restricted access to groundwater. The stygobite database available for the UK dating back to the early 1900's is a good example for this, because samples were often obtained opportunistically (e.g. in Highly Karstic Limestone), collected with differing sampling methods and heavily biased to certain geologies (e.g. the Chalk). However, the recent decade has seen a shift towards more standardised and replicable sampling designs in groundwater ecology research. Genrally, four common sampling designs in field studies are regular sampling, random sampling, equal random-stratified sampling and proportional random-stratified sampling (Hirzel & Guisan, 2002), with the latter usually being chosen in groundwater studies. The PASCALIS study, a trans-national project to understand distribution controls on groundwater ecosystems, was the first effort to employ a standardised sampling design with a total of 1152 randomly selected sites (Dole-Olivier et al., 2009; Galassi et al., 2009; Gibert et al., 2009). More recently, a study undertaken in Devon and Cornwall (England) was the first to use stratified sampling in the context of a more detailed habitat typology (Johns et al., 2015).

The observed patterns of biodiversity are also often related to sampling effort (Tomlinson & Boulton, 2010), meaning that distribution data should always be interpreted with caution. For example, a single net sample may only collect 23 % of species, increasing to 38 % in the second net haul (Eberhard et al., 2009), suggesting that multiple net hauls are needed to detect species that occur in low abundances. Due to the variable quality of geo-habitats (chapter two) and the patchy distribution of stygobites (chapter four), it is also essential to have good regional sampling coverage to detect rare species. False-negatives resulting from insufficient sampling replication (both within and between-site) pose serious problems for biogeographical studies (Castellarini et al., 2007; Hancock & Boulton, 2009) and targeted conservation planning (EPA, 2003). A good example for this in the UK is *A. stammeri*, which may escape net hauls because it occurs in low abundances. It is also a rare species indicating that the relatively low sampling coverage of northern England and Wales (Robertson et al., 2009) may be insufficient to detect it.

Several aspects of this thesis deal with issues of sampling designs and sampling bias. The geo-habitat typology developed in chapter two provides a sampling framework that minimises the number of habitats needing to be sampled, while maximising the hydrogeological explanatory power needed to assess the controls of species distributions at a national scale. Combining this framework with appropriate sampling replication and coverage in the geo-habitats, could provide future studies with the tools to better evaluate distribution controls. The sampling bias in England and Wales (almost 50 % from the Chalk) remains one of the main limitations to understanding species distributions. To start addressing this issue I undertook the first sampling in two sandstone geologies (Devonian Old Red Sandstone, Lower Greensand) and extended the sampling coverage of

two limestones (Jurassic Limestone, Magnesian Limestone). However, this is only a first step towards a more balanced ecological dataset and future studies are needed to improve geological and geographical sampling coverage (see section 6.7).

6.5 Stygobites may influence ecosystem processes

The two grazing experiments conducted for chapter five showed that stygobite presence significantly increased protozoan abundance and diversity, and changed bacterial community composition (illustrated in Fig. 6.1; Weitowitz et al., submitted b). Although the underlying mechanisms for this were not identified, it seems most probable that stygobite bioturbation enhances the availability of food (i.e. bacteria) to protozoans. This most likely occurs through a combination of scraping off and dislodging bacterial biofilm. For example, crustacean bioturbation is a major modifier of hyporheic (Barlocher & Murdoch, 1989) and surface water biofilm (Laverock et al., 2010), and may have a similar effect in groundwater (Sinton, 1984; Boulton et al., 2008; Robertson et al., 2009). Stygobites may be part of a positive feedback loop that increases the number and diversity of protozoans, which in turn enhance bacterial activity rates that control the provision of ecosystem services (e.g. water purification, contaminant attenuation; Griebler & Avramov, 2015) benefitting humanity (see Daily, 1997).

The chemical reactions underlying these services are primarily carried out by microbes (Diaz et al., 2013; Jeon & Madsen, 2013; Griebler & Avramov, 2015) and a regulatory link between protozoa and bacteria is well established (Sanders & Wickham, 1993; Kota et al., 1999; Langenheder & Juergens, 2001; Pernthaler, 2005). Furthermore, protozoa are known to accelerate many bacteria-mediated decontamination processes

(Biagini et al., 1998; Hahn & Hoefle, 2001; Mattison et al., 2002; Mattison & Harayama, 2005; Humphreys, 2008). This may indicate that ecosystem services are most efficiently rendered where stygobites are present. However, increased protozoan grazing can also inhibit bacterial activity, as shown by reduced hydrocarbon-degradation in another microcosm experiment (Beaudoin et al., 2016).

The two complementary experiments illustrate that, despite their truncated nature, groundwater food webs may have considerable complexity. This is also corroborated by the results from the second experiment, which demonstrated that both time and grazer density are important factors in mediating top-down grazing effects by stygobites. The importance of such factors has been well established for crustaceans in surface waters (Adrian & Schneider-Olt, 1999; Engkvist et al., 2000), but experimental evidence is scarce for groundwater ecosystems. My experiments do not provide evidence for the popular notion that stygobites directly affect ecosystem services (Boulton et al., 2008; Tomlinson & Boulton, 2010), because I did not identify particular bacterial strains or their metabolic activity. However, they clearly indicate that stygobites actively modify their environment, despite possessing adaptations (e.g. low metabolic activity) to the low nutrient environment (Gibert et al., 1994). Stygobites have been described as potential ecosystem service facilitators (ESFs) with multiple ecological roles (Boulton et al., 2008), and chapter five provides additional support for this.

When considering the role of stygobites as grazers in groundwater ecosystems, three parameters may be important in conveying their effects: Stygobite occurrence (distribution of ‘complex’, fully functional ecosystems), diversity (number of species that contribute to ecosystem services and / or provide redundancy; experiment one) and

abundance (threshold density that may be necessary; experiment two). All of these parameters may be strongly affected by anthropogenic pollutants, such as pesticides and heavy metals (Di Marzio et al., 2008), nitrates (Di Lorenzo & Galassi, 2013), and excessively high levels of organic matter (Datry et al., 2005; Hahn, 2006). Groundwater food webs are truncated with few trophic components (Gibert & Deharveng, 2002) and therefore all organisms (microbes, protozoa, invertebrates) are likely to fulfil crucial roles (Boulton et al., 2008). A growing body of research suggests a positive link between biodiversity and ecosystem function (e.g. Mittelbach et al., 2001; Duffy et al., 2003; Balvanera et al., 2006), highlighting that low biodiversity ecosystems are more affected by species loss, because they have much lower functional redundancy (Lawton & Brown, 1994; Allison & Martiny, 2008; Reich et al., 2012).

While research in surface ecosystems has moved on to exploring the mechanisms that link biodiversity and ecosystem function (Reiss et al., 2009), much of the groundwork remains to be done for groundwater ecosystems (e.g. what ecosystem processes / services may be affected by grazers). Chapter five elucidates the role of these unique grazers in their ecosystems and highlights their potential involvement in the provision of their groundwater ecosystem services. Overall, knowing the distributions of complex groundwater ecosystems at the large-scale (Fig. 6.1; chapter four) is important to understand where ecosystem services may be carried out most efficiently. However, the trophic interactions investigated in chapter five occur in individual sections of the aquifer or in individual boreholes at the small-scale (Fig. 6.1). The significance of these interactions depends on the abundance and biomass of stygobites in aquifers, which remain poorly known (Maurice & Bloomfield, 2012; but see Hahn & Matzke, 2005; Sorensen et al., 2013), but are likely to be lower than in boreholes.

6.6 Conservation of groundwater ecosystems

One of the primary reason for conserving groundwater ecosystems is that they contribute considerably to the global pool of biodiversity. While 6,634 aquatic stygobite species have been described (Botosaneanu, 1986), this is probably a gross underestimate of the global stygobite biodiversity. Furthermore, globally groundwater habitats have some of the highest proportions of endemic and narrowly distributed taxa (Gibert & Deharveng, 2002), with many species limited to single sites. However, groundwater ecosystems in the UK are composed of very few species, indicating they might be particularly vulnerable to community changes and highlighting the urgent need for the holistic protection of these systems. There is also growing recognition that groundwater ecosystems form essential components of the domain that provides potable water to humanity (Tomlinson & Boulton, 2008; Griebler et al., 2010), and ultimately determines the integrity of surface water ecosystems (Boulton, 2005).

Numerous field studies and experiments have shown that stygobites may be impacted by human activity altering water temperatures, hydrochemical parameters and aquifer water tables (e.g. Notenboom et al., 1995; Mösslacher, 2000; Canivet et al., 2001; Scarsbrook & Fenwick, 2003; Brielmann et al., 2009; Tomlinson & Boulton, 2010; Di Lorenzo et al., 2014, 2015). For example, microcosm experiments showed that while meiofauna (e.g. copepods) are able to track declining water tables, the larger amphipods become stranded (Tomlinson & Boulton, 2010). Furthermore, organic pollution has been linked with decreased stygobite diversity and abundance (Notenboom et al., 1995; Datry et al., 2005), although this is likely to be species-specific (Scarsbrook & Fenwick, 2003). Chapters three and four provide further evidence for the low dispersal capacities of

stygobites, highlighting their particular vulnerability to anthropogenic disturbance, because they may require long periods of time for recolonization.

Both the hydrochemical tolerances (Di Lorenzo & Galassi, 2013; Iepure et al., 2013; Marmonier et al., 2013; Di Lorenzo et al., 2015) and recolonization potentials differ between species, as demonstrated by the apparently very different dispersal capacities of e.g. *N. aquilex* and *N. glenniei* discussed in chapter four. Species-specific susceptibilities and behaviours are used to tailor conservation policies in surface ecosystems (Blumstein et al., 2003; Cooke & Suski, 2005; Pressey et al., 2007), and the same is undoubtedly required for groundwater ecosystems.

In 2000 the EU Water Framework Directive still distinguished between surface ecosystems and the groundwater realm, stating the need for protection of surface ecosystems, while solely focussing on good chemical status of the latter. Both in groundwater research (e.g. Korbel & Hose, 2011) and in the policy of some governments, this notion has shifted to a more holistic notion of groundwater acknowledging their resident ecosystems. For example, the Swiss Water Protection Ordinance dispensed in 1998 specified that groundwater biota should be maintained in a natural state reflecting low pollution levels (Goldscheider et al., 2006). A policy released in New South Wales, Australia, highlighted the need for the maintenance and restoration of ecological processes and biodiversity in aquifers (Griebler et al., 2010), while the Western Australian Protection Authority developed guidelines for assessing the impact on stygobites when extracting groundwater (EPA, 2003).

The EU Groundwater Directive (EU-GWD, 2006) stipulates that ‘research should be conducted in order to provide better criteria for ensuring groundwater ecosystem

quality'. However, the recognition of *N. glenniei* as a Biodiversity Action Plan species (Knight, 2009) aside, little has been done to incorporate groundwater ecosystems into policies in the UK. Chapter four showed that the highest biodiversity in England and Wales is found in karstic habitats, such as the Chalk, Highly Karstic and Moderately Karstic Limestone. Due to their high permeability and the fact that they are often located in heavily populated areas, many of these species-rich habitats are also the most susceptible to pollution (e.g. see bromate plume in the Chalk, Cook et al., 2012; nitrate efflux from agriculture, Wang et al., 2013). To keep up with policy development elsewhere and in recognition of the growing threats to groundwater ecosystems, there is a need for incorporating at least some ecological aspects into future iterations of the GWD (EU-GWD, 2006).

Identifying conservation priorities (e.g. focusing on rare species or biodiverse habitats) is challenging, because ecosystem disturbances are often geographically far-reaching and transcend habitat boundaries (Kundzewicz et al., 2008; Green et al., 2011). However, due to a lack of time and financial resources, it is not possible to protect all organisms and all habitats simultaneously (Gibert et al., 2009). Traditionally, biodiversity hotspots have often been prioritised in conservation research (Randall, 1991; Boulton et al., 2003; Turpie, 2003; Culver & Sket, 2000; Michel et al., 2009). Nevertheless, there is also a strong argument for protecting low-diversity ecosystems, such as Igneous and Metamorphic Rock discussed in chapter four. Further species loss has profound negative consequences on the functioning of such ecosystems, because there are few, if any, alternative species that can carry out the functional roles of lost species (Naeem & Li, 1997; Micheli & Halpern, 2005).

In article 20 the EU Groundwater Directive (2006) states that "[…] the findings obtained [in groundwater ecosystem research] should be taken into account when implementing or revising this Directive". While targeted conservation efforts may be necessary to protect certain species or aquifers in the short-term, it is holistic policies addressing both the abiotic and biotic components of groundwater that are the key for successful conservation at large spatial scales and over longer time periods (see section 6.7).

6.7 Future research

Improving our knowledge of groundwater ecosystem distributions is of primary importance both within the UK and globally, because it informs all other areas of groundwater research. The unexpectedly low stygobite diversity and frequency of occurrence in some geo-habitats (e.g. northern outcrop of Moderately Karstic Limestone, Mildly Karstic Limestone) indicates that more sampling is required to verify this pattern. Little is known about the importance of porous (in the UK) and fractured geologies (globally) for groundwater ecosystems, even though they dominate the subterranean landscape of many countries (e.g. Cornu et al., 2013; Weitowitz et al., submitted a). As outlined in chapters three and four, some fractured geo-habitats in England and Wales harboured notable stygobite assemblages, highlighting the need for a better sampling coverage of fractured aquifers to complement the limited number of studies already conducted in these systems (Hahn & Fuchs, 2009; Larned, 2012; Johns et al., 2015). Furthermore, porous bedrock has not been sampled in the UK, although this may be a highly suitable habitat where pore spaces are sufficiently large for fauna (e.g. Large-Pore

Unconsolidated in chapter two). Given its probable suitability to stygobites, the complete lack of faunal data for porous rocks in the UK is probably the most significant gap in our knowledge of UK species distributions.

It would also be useful to confirm whether the low stygobite diversity in northern England (but south of the glacial limit) has been shaped by past periglacial conditions, in order to determine how far south the Devensian glacial ice sheet affected stygobite populations. A useful approach would be to consider studies that model historical permafrost depths in the UK during the Devensian (e.g. Busby et al., 2015). Two regions with and without permafrost cover within the same geology (e.g. Chalk) could be selected for comparison, in order to remove biasing effects of geology. These different permafrost thicknesses could then be linked to present-day stygobite communities in those areas, for example by comparing stygobite species diversity, abundances or frequencies of occurrence.

My habitat typology in chapter two was verified using hydrogeological and hydrochemical data from the UK, and tested on faunal data at a large spatial scale (England and Wales). Ecological data collected and analysed within this framework (or a modified version thereof) in other regions, may enable a better assessment of geological controls at national scales. Because it incorporates higher geological resolution, it may provide higher explanatory power on broad spatial scales, compared to traditional, more broad typologies. Furthermore, the use of more detailed typologies may better contribute to the incorporation of ecological criteria in conservation policy. For example, ecological parameters (e.g. species diversity and frequency of occurrence) from each geo-habitat could be used to set target values for aquifers to maintain ecosystem integrity, as

highlighted in article 20 of the Groundwater Directive. The invertebrate assemblages discussed in chapters three and four are only one important component of groundwater ecosystems. Future studies should also assess other biotic components integral to groundwater ecosystem functionality in the context of more detailed typologies, including invertebrate activity (Sinton, 1984; Malard et al., 1996), microbial diversity and activity (Mermillod-Blondin et al., 2000; de Lipthay et al., 2004) and trophic relationships (Costanza & Mageau, 1999).

Geological variability was found to influence groundwater fauna at both the mid (aquifer) scale and the small (borehole / site) scale. For example, my typology did not explain the high species diversity in Mudstones & Siltstones in chapter four, nor could geology account for copepod biomass and abundance in chapter three. Both of these issues probably relate to site-specific geological features (Sorensen et al., 2013). For example, a borehole in Mudstones & Siltstones intercepting limestone strata is likely to contain more stygobites and higher copepod biomass than a borehole in an area of low fracture density. Future regional and local scale studies need to consider geology in more detail to disentangle faunal distribution and biomass patterns at these respective scale. I consulted British Geological Survey borehole scans to help in identifying the major geological formations at boreholes (http://www.bgs.ac.uk/data/boreholescans), but nested within this remains important lithological detail. 3d scans of boreholes showing the consolidation of surrounding rock, and the size and density of fractures may be extremely helpful in better understanding the ecology of individual sites and whole aquifers. However, currently such scans are still time-consuming, costly to undertake and only available for few locations (see Sorensen et al., 2013).

More targeted experiments are needed to follow on from my grazing experiments in chapter five, to assess a possible link between stygobites and ecosystem services. In my experiments I distinguished bacteria on cell size and protozoans by morphotype, but using specific bacterial strains or protozoan species may improve the meaningfulness of future experiments. For example, previous studies have identified the specific microbial strains involved in the degradation of hydrocarbons (Rabus & Widdel, 1995), the decontamination of heavy metal polluted water (Mehrotra et al., 2016) and the denitrification of groundwater (Zhang et al., 2015). Known bacterial strains could be isolated and combined with different protozoan assemblages, stygobite species and densities in factorial experiments to measure the effect on a desired response variable, such as a pollutant. In my experiments I also could not verify that stygobites actively graze on biofilm, because I could not be sure whether the initial change in bacterial community structure was due caused by the invertebrates or protozoan grazing. Designing experiments with treatments of consisting of all trophic components (bacterium, protozoan, grazer) individually and in all possible combinations, would enable a clear identification of the causal links in the microcosms.

6.8 Conclusion

Ricklefs et al. (1984) highlighted that the “first concern of environmental management based on ecological processes must be the integrity of the water system”. The groundwater domain has a global significance for both animals and humans, harbouring high biodiversity (Botosaneanu, 1986; Danielopol et al., 2000; Maurice & Bloomfield, 2012), and providing clean drinking water and other ecosystem services (sensu Daily,

1997; Dudgeon et al., 2006; Griebler & Avramov, 2015). The status of groundwater ecosystems also affects the integrity of surface water bodies because of their linkages, and exchanges of nutrients and organic matter along ecotonal boundaries (Gibert et al., 1990).

My thesis contributes to an increased understanding of the factors controlling these ecosystems and groundwater ecosystem functionality. It highlights that geology and its associated variability are key determinants of stygobite communities at all spatial scales, ranging from individual boreholes at the small-scale to the large-scale distribution across England and Wales. I propose my new habitat typology as a framework with higher geological explanatory power for assessing stygobite species distributions at the national scale. I develop habitat quality scores that incorporate the hydrogeological and hydrochemical variability of geo-habitats, and propose that they may be a useful way of predicting habitat suitability to groundwater ecosystems. Using the new typology to analyse stygobite distributions, indicates that while geology is important, glacial history, slow dispersal, and local site characteristics often supersede its effect. Finally, the grazing experiments show that stygobites themselves affect other ecosystem components (protozoa and bacteria) at the small-scale, potentially influencing the provision of ecosystem services across aquifers.

Despite a growing interest in groundwater ecology, this field is still at an early developmental stage compared to many well-known surface ecosystems (Larned, 2012). Therefore, further observational studies, manipulative field experiments and laboratory experiments are needed to better understand all aspects of groundwater ecosystems, including their distribution controls and functional ecology. These unique ecosystems are

under mounting pressure from growing populations and increasing levels of anthropogenic pollution. Further research on groundwater ecosystems and their legal protection should therefore be pursued swiftly, while these ecosystems remain functional and human impact can be minimised.

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